# *Journal of*
# DAIRY SCIENCE

Vol. 42 November, 1959 Number 11


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DIRECTORS OF A.D.S.A.

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# JOURNAL OF DAIRY SCIENCE

OFFICIAL PUBLICATION OF THE
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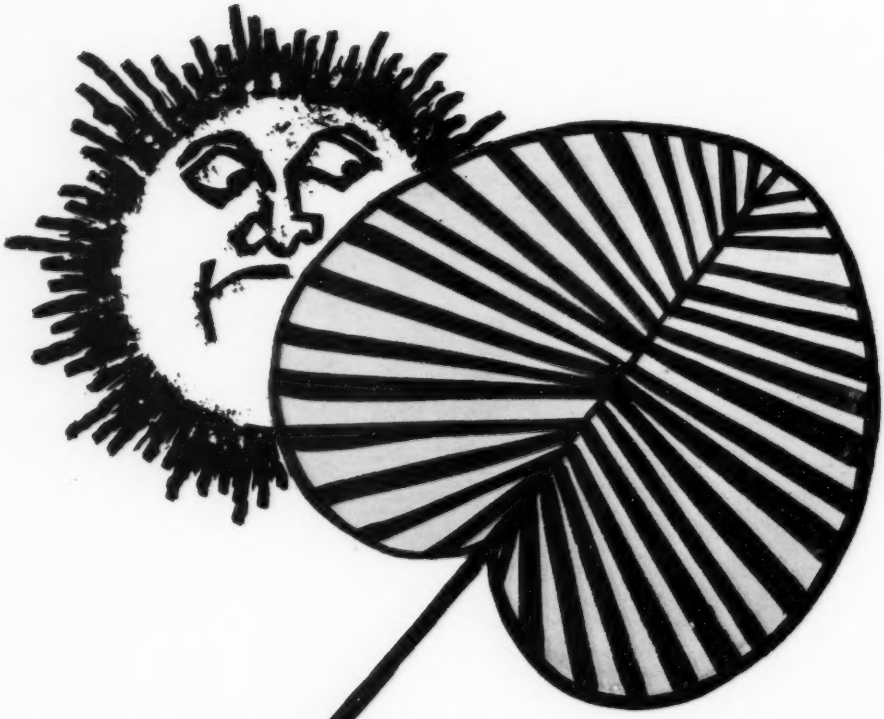

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# PEOPLE AND EVENTS

## COLLEGE & UNIVERSITY NEWS

### Illinois, Iowa State, Kansas State Teams Win in Dairy Products Judging Contest; Three Fellowships Granted

Three men from the University of Illinois covered themselves with glory and the trophy rooms of their alma mater with cups by their sterling performances in the 25th Collegiate Students' International Contest in Judging Dairy Products, held during October in Miami Beach.

When winners were announced on Tuesday evening, October 6, three of the five team awards were captured by the three-man team of E. A. LAWTON, JR., Dixon, Ill.; J. A. MERRIELL, Clinton, Ill.; and R. L. SCHWARBERG, Springfield, Ill. The three: All Products Bowl, Cheese Cup, and Butter Cup. The Bowl carries with it a cash fellowship for $1800, which will enable one of the team to undertake a year of graduate work. JOSEPH TOBIAS was the faculty coach who trained the judging champs.

Two of the Illinois team members ranked high in individual categories. Schwarberg was the highest ranking judge of all products, and received a watch for his work. Merriell took the bronze medal for third place in cheese judging.

Iowa State University was the second-top team in judging all dairy products, and the team coached by W. S. ROSENBERGER received a $1650 cash fellowship. The team members were G. K. BROADY, Sewell, N.J.; V. L. LAUN, Ames, Iowa; and J. F. SINKLER, Nevada, Iowa.

Two Iowa State boys also ranked high in individual scoring: Sinkler was first in ice cream judging and received a watch for his performance. Broady was the second-highest ranking individual judge of all products, and received a silver medal.

Third ranking team in judging all products came from the Kansas State University where they had been coached by ROSS MICKELSEN, and their efforts earned them a $1500 cash fellowship. The three-man team was composed of C. D. FREDERICK, Parsons, Kan.; DEAN GIGSTAD, Nortonville, Kan.; and D. I. VINCENT, Manhattan, Kan.

Two individuals from Kansas received individual recognition for single-product performances. Vincent was named second-best butter judge and was awarded a silver medal. Gigstad was third in milk and received a bronze medal.

A total of 24 teams from every part of the United States entered this 25th staging of the international judging classic, jointly sponsored by American Dairy Science Association and Dairy Industries Supply Association. Winners were presented their awards at a gala South Seas Festival and luau, held at the Roney Plaza.

Industry sponsors of awards are: Milk Industry Foundation — the milk individual awards and team cup; International Association of Ice Cream Manufacturers — the ice cream individual awards and team cup; American Butter Institute — the butter individual awards; National Cheese Institute—the cheese individual awards; Dairy Industries Supply Association — the all products individual awards and team bowl, the cheese cup, the butter cup, and all cash fellowships.

Contest superintendent this year was L. H. BURGWALD, Marketing Specialist, Dairy and Poultry Division, Foreign Agricultural Service, U. S. Department of Agriculture, Washington, D.C. Assistant superintendent was D. R. STROBEL, Deputy Director, Foreign Marketing Branch, Dairy and Poultry Division, Foreign Agriculture Service, U. S. Department of Agriculture, Washington, D.C.

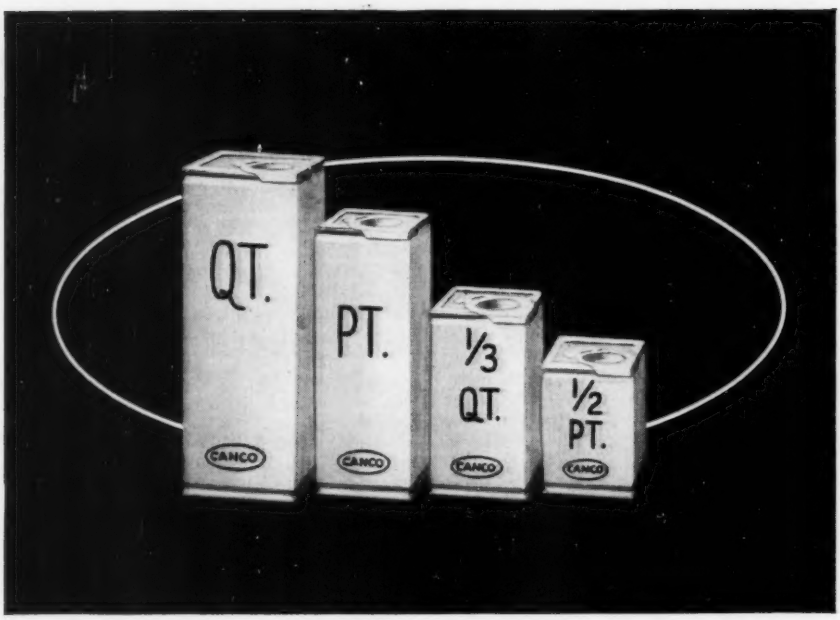
Official judges of products were: N. E. FABRICIUS, Flambeau Valley Farms Cooperative, All-Products; D. A. PETTEE, The Creamery Package Mfg. Company, Milk; H. L. WILSON, Consolidated Dairy Products Co., Cheese; J. H. ERB, The Borden Company, Ice Cream; B. J. ALDRICH, H. C. Christians Co., Butter.

Participating schools were: California State Polytechnic College, Clemson Agricultural College, Colorado State University, University of Connecticut, University of Florida, University of Georgia, University of Illinois, Iowa State University, Kansas State University, University of Kentucky, Michigan State University, University of Minnesota, Mississippi State University, University of Misouri, North Carolina State College, Ohio State University, Oklahoma State University, The Pennsylvania State University, Purdue University, University of Rhode Island, Rutgers University, Texas Technological College, Virginia Polytechnic Institute, West Virginia University.

Below are the top ten teams and individuals in all categories, along with their official scores, in this annual contest.

**All Products**

| TEAM | SCORE |
|---|---|
| 1. Illinois | 13 |
| 2. Iowa | 14 |
| 3. Kansas | 19 |
| 4. Minnesota | 29 |
| *5. California | 33 |
| *5. Mississippi | 33 |
| 7. Oklahoma | 35 |
| 8. Ohio | 38 |
| 9. Connecticut | 43 |
| 10. Georgia | 44 |




# MILK

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| INDIVIDUALS | SCORE |
|---|---|
| 1. R. L. Schwarberg, Illinois | 37 |
| 2. G. Broady, Iowa | 45 |
| 3. R. G. Cox, Mississippi | 56 |
| *4. V. L. Laun, Iowa | 59 |
| *4. P. J. Fitzsimmons, Oklahoma | 59 |
| 6. D. E. Lord, California | 61 |
| 7. J. A. Merriell, Illinois | 66 |
| 8. C. D. Frederick, Kansas | 73 |
| 9. J. T. Stork, Minnesota | 75 |
| 10. D. D. Vincent, Kansas | 84 |

* Tie

**Milk**

| TEAM | SCORE |
|---|---|
| 1. Ohio | 48.74 |
| 2. California | 49.33 |
| 3. Iowa | 50.82 |
| 4. Kansas | 51.50 |
| 5. Michigan | 52.67 |
| 6. Georgia | 53.00 |
| 7. North Carolina | 53.58 |
| 8. Illinois | 53.82 |
| 9. Oklahoma | 54.41 |
| 10. West Virginia | 55.51 |

| INDIVIDUALS | SCORE |
|---|---|
| 1. M. Vander Sloot, Michigan | 13.08 |
| 2. D. R. Heldman, Ohio | 13.33 |
| 3. D. E. Gigstad, Kansas | 14.83 |
| 4. R. V. West, Ohio | 15.08 |
| *5. P. Howse, Georgia | 15.17 |
| *6. F. Jacinto, California | 15.17 |
| 7. M. G. Adams, Jr., North Carolina | 15.50 |
| 8. R. Gayauskas, California | 15.83 |
| 9. G. Broady, Iowa | 16.24 |
| 10. R. L. Schwarberg, Illinois | 16.33 |

* Tie broken on flavor score

**Cheese**

| TEAM | SCORE |
|---|---|
| 1. Illinois | 102.90 |
| 2. Kansas | 105.35 |
| 3. Ohio | 106.17 |
| 4. California | 106.46 |
| 5. Minnesota | 109.35 |
| 6. Mississippi | 109.52 |
| 7. Iowa | 110.71 |
| 8. Kentucky | 111.52 |
| 9. Pennsylvania | 112.44 |
| 10. Georgia | 114.11 |

| INDIVIDUALS | SCORE |
|---|---|
| 1. D. E. Lord, California | 30.20 |
| 2. P. L. Culler, Ohio | 31.06 |
| 3. J. A. Merriell, Illinois | 31.30 |
| 4. R. L. Schwarberg, Illinois | 32.76 |
| 5. R. G. Cox, Mississippi | 33.01 |
| 6. C. D. Frederick, Kansas | 33.67 |
| 7. M. E. Howell, North Carolina | 33.97 |
| 8. G. Behrens, Minnesota | 34.34 |
| 9. P. J. Fitzsimmons, Oklahoma | 34.42 |
| 10. D. Toloday, Purdue | 34.67 |

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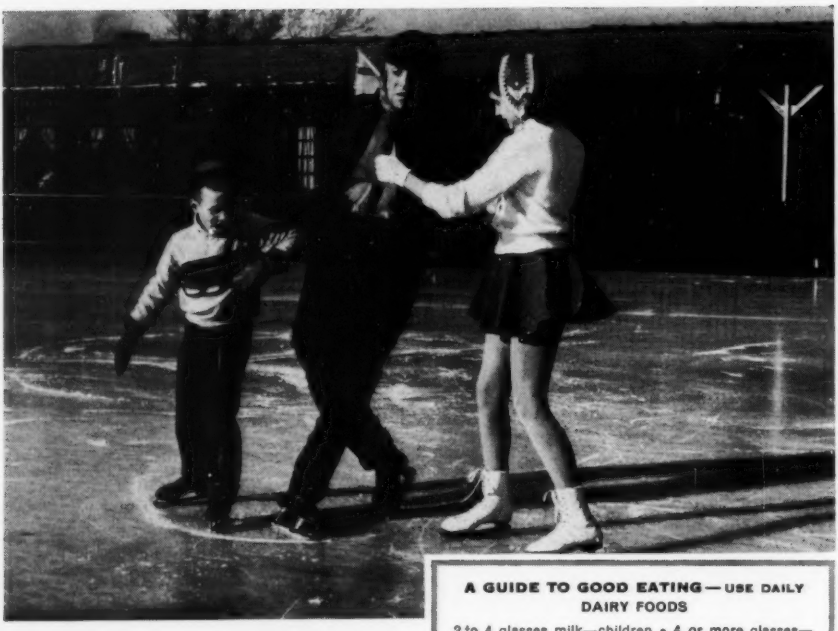

# CONTROL

Foods can provide many minerals needed for control of body functioning . . . as well as for structure of body tissues . . . but special consideration needs to be given to insure that the diet contains enough of certain minerals.

The minimum quantities of foods listed in *A Guide to Good Eating* can provide most of the Recommended Dietary Allowances for *calcium . . . 2/3 from milk group . . . and ¼ from other groups.*
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2 or more servings • Meats, fish, poultry, eggs, or cheese—with dry beans, peas, nuts as alternates

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### Butter

| TEAM | SCORE |
|---|---|
| 1. Illinois | 36.68 |
| 2. Iowa | 37.89 |
| 3. Minnesota | 39.92 |
| 4. Kansas | 43.67 |
| 5. Mississippi | 44.17 |
| 6. Purdue | 47.18 |
| 7. California | 47.84 |
| 8. Michigan | 48.92 |
| 9. Oklahoma | 51.01 |
| 10. Pennsylvania | 54.01 |

| INDIVIDUALS | SCORE |
|---|---|
| 1. D. Grunnes, Minnesota | 6.67 |
| 2. D. D. Vincent, Kansas | 7.25 |
| 3. D. E. Lord, California | 8.67 |
| 4. V. L. Laun, Iowa | 8.80 |
| 5. D. Webb, Jr., Mississippi | 11.50 |
| 6. R. L. Schwarberg, Illinois | 11.67 |
| 7. E. A. Lawton, Jr., Illinois | 11.84 |
| 8. R. K. Brinegar, Purdue | 12.67 |
| 9. J. A. Merriell, Illinois | 13.17 |
| 10. G. Broady, Iowa | 13.42 |

### Ice Cream

| TEAM | SCORE |
|---|---|
| 1. Minnesota | 123.34 |
| 2. Iowa | 123.81 |
| 3. Illinois | 129.26 |
| 4. Oklahoma | 131.84 |
| 5. Connecticut | 132.17 |
| 6. Georgia | 132.76 |
| 7. Colorado | 134.59 |
| 8. Purdue | 138.18 |
| 9. Kansas | 138.51 |
| 10. Mississippi | 138.67 |

| INDIVIDUALS | SCORE |
|---|---|
| 1. J. F. Sinkler, Iowa | 35.80 |
| 2. P. J. Fitzsimmons, Oklahoma | 39.00 |
| 3. R. G. Cox, Mississippi | 39.17 |
| 4. D. Grunnes, Minnesota | 39.50 |
| 5. J. L. Rundell, Michigan | 40.17 |
| 6. J. S. Payne, Georgia | 40.50 |
| 7. J. T. Stork, Minnesota | 41.34 |
| *8. C. W. Coblentz, West Virginia | 41.50 |
| *9. R. T. Middlebrook, Connecticut | 41.50 |
| 10. J. A. Merriell, Illinois | 41.76 |

* Tie broken on flavor score

### Florida News

P. T. D. ARNOLD, associate dairy husbandman and associate professor of dairying, retired last June after serving the University of Florida for 28 years. He has written extensively in the field of dairy herd management and has earned the reputation of being an excellent teacher. He will continue to live in Gainesville.

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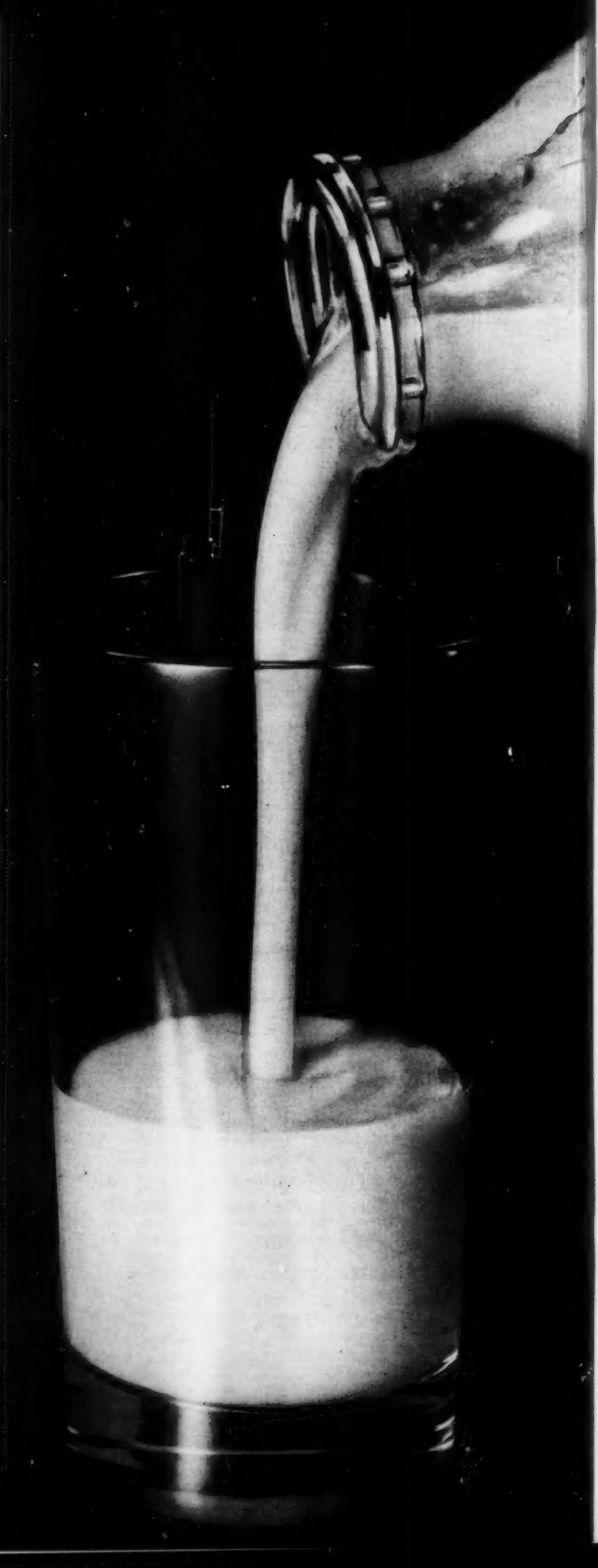

C. J. WILCOX, who recently earned a Ph.D. from Rutgers University has joined the Florida staff to replace Prof. Arnold. Dr. Wilcox graduated from the University of Vermont in 1950, served two years in Korea with the U. S. Army, received his M.S. from Rutgers in 1955, and operated his own dairy farm for two years. He will be assistant dairy husbandman and assistant professor of dairying.

---

D. H. KLEYN, who earned his M.S. from Ohio State University in 1953, has been named assistant professor of dairy manufacturing and assistant dairy technologist at Florida. He replaces B. J. LISKA, who resigned last June to accept a position at Purdue University.

### Kentucky News

W. S. LAGRANGE has been appointed Extension Technologist in Dairy Manuafacturing for the Dairy Section of the University of Kentucky.

Dr. LaGrange fills a new position created in the Dairy Section. He will institute a program of quality control and improvement for Kentucky market milk and ice cream plants. Much of his work will be in the field contacting and advising the various plants as well as instituting and supervising a laboratory at the University to be used in this work.

Born in Ames, Iowa, in 1931, Dr. LaGrange earned the B.S. degree from Iowa State College in 1953. He completed his work for the Ph.D. degree in 1959 at the same institution, specializing in dairy bacteriology and dairy economics. He has given several papers before the Society of American Bacteriologists and the American Dairy Science Association.

He is a member of the A.D.S.A., Society of American Bacteriologists, International Association of Milk and Food Technologists, Alpha Zeta, Gamma Sigma Delta, and Seabbard and Blade.

### News from Ohio

I. A. GOULD, chairmen of the Department of Dairy Technology, attended the 15th International Dairy Congress in London as an advisory delegate selected by the USDA. While in Europe, Dr. Gould visited with research leaders and educators at Hannah Dairy Institute, Scotland; Dairy Research Institute, Kiel, Germany; Royal Agricultural College, Copenhagen, Denmark; Dairy Research Institute, Hilleroed, Denmark; Agricultural Research Institute, Alnarp, Sweden; and the Agricultural College, Volebek, Norway. He also visited major dairy plant operations in London, Copenhagen, Stockholm, and Oslo.


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(1) Standard Methods for the Examination of Dairy Products, ed. 10, New York, American Public Health Association, 1953. (2) Committee Report, Am. J. Pub. Health 42:1131 (Sept.) 1952. (3) Microbiological Methods, report at 66th Ann. Meet. Assn. Official Agricultural Chemists, Sept. 29, 1952: J. Assn. Official Agr. Chem. 36:91 (Feb.) 1953. (4) Standard Methods for Examination of Water, Sewage and Industrial Wastes, ed. 10, New York, American Public Health Association, 1955.

Literature on B-B-L's **complete** line of culture media and apparatus for the microbiological laboratory sent on request. (13)

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Winners of the 1959 Ohio State Fair Dairy Products Contest have been announced by the Ohio State Fair Dairy Project steering committee and Ohio State University. They are as follows:

Swiss Cheese, Wheel—First, ADEL LADRACH, Sugar Creek; second, ERNEST STALDER, Pearl Valley; third, FOREST HICKS, Ragersville.

Swiss Cheese, Rindless Block—First, HANS JORG, Pleasant View; second, ERVIN FIENE, Kidron; third, ADEL LADRACH, Sugar Creek.

**J. M. Jensen, Michigan State University (rear, right) discusses Cottage cheese quality with participants in a clinic held in conjunction with the 1959 Ohio State Fair Cottage Cheese Makers Competition.**

Milk Judging—First, B. E. SHIVELY, Columbus; second, DAVID MIZER, Cadiz; third, FRED CARVER, Columbus.

Ice Cream Judging—First, FRED CARVER, Columbus; second, BERT TAYLOR, Columbus; third, C. T. ABELL, Medina.

Cottage Cheese — First, CLYDE DE LONG, Grove City; second, PAUL LUYSLER, Barnesville; third, CHARLES WOLFORD, Columbus.

In addition to cash prizes and ribbons, the three winners in each of the milk and cottage cheese contests received certificates presented jointly by the Milk Industry Foundation.

Official judges of the contest were R. E. HARDELL, Monroe, Wis., and J. M. JENSEN, Michigan State University.

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The 11th Annual Dairy Technology Homecoming Brunch was held in October. Classes featured this year were 1959, 1949, 1939, 1929, 1919, and 1909.

## GOVERNMENT NEWS

### Dairy Society International

The board of directors of Dairy Society International re-named as its officials for the 1959-60 period those who had served for 1958-59.

These were MILTON HULT, president of National Dairy Council, Chicago, re-elected chairman of the board; GORDON LAMONT, Darien, Conn., vice-chairman; ROBERTS EVERETT, managing director; N. E. QUIBLE, assistant treasurer; and GERTRUDE C. KALEC, assistant secretary.

Re-appointed to the Society's executive committee, in addition to Hult and Lamont were: M. J. FRAMBERGER, American Dairy Association, Chicago; WILLIAM HITZ, Iowa State Dairy Association, Polk City; L. D. MCKEE,

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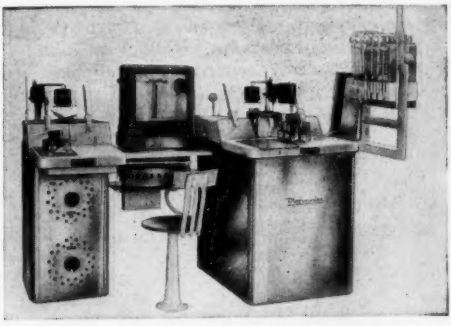

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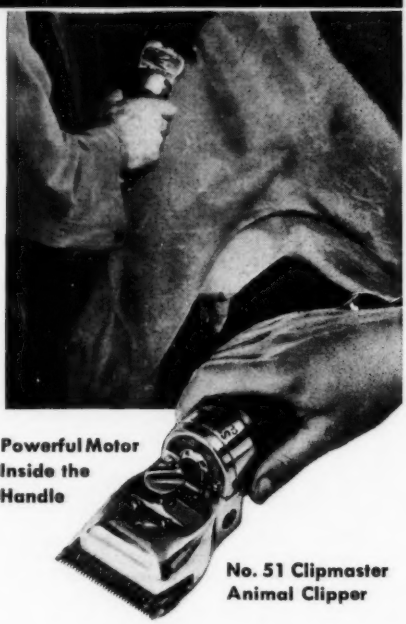


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Leading health authorities say: "A regular clipping program means more wholesome milk. It is an essential step in the production of quality dairy products." Emphasize the advantages of regular clipping. It reduces sediment, lowers bacteria, avoids contamination and increases profits from production of cleaner, higher quality milk.

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The XV International Dairy Congress was held in London, June 29-July 3, 1959 under Royal Patronage and it was generally acclaimed as a very successful scientific and social event. At this Congress 380 papers were discussed from the world's dairy scientists, technologists, engineers, economists and government officials. Together with the invited lectures, symposium speeches, verbatim reports of the discussions and an illustrated account of the whole Congress, these 380 papers are published in the Proceedings, which extend to five volumes.

The first four volumes have already been printed and are bound in blue cloth with gold blocking and contain the 380 papers together with summaries in English, French and German. The fifth and final volume will be issued early in 1960.

A limited number of sets of these volumes are now available in addition to those given to registered Congress members. They can be obtained at 8 pounds per set of five volumes from the Congress Secretariat in London. Orders should be sent immediately to:

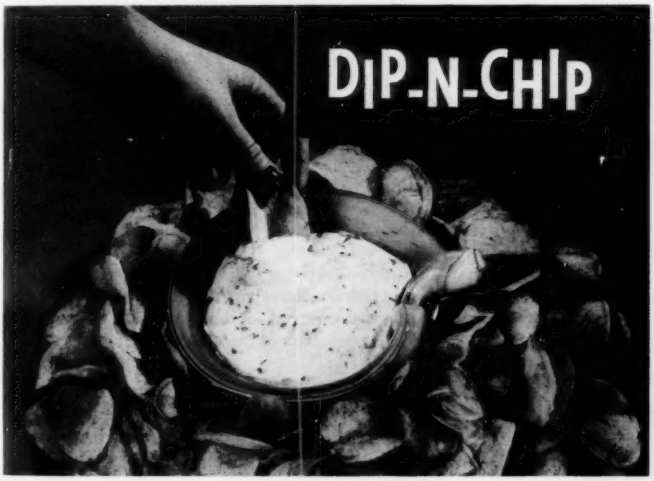
A. W. MARSDEN, Organizing Secretary, XV International Dairy Congress, 86 Brook Street, London, W.1., England. Checks, drafts, or money orders should be made payable to "XV International Dairy Congress" and should be in sterling and not in dollars, marks, francs or other currency.

Each set of five volumes is 8 pounds post free. After the present issue is exhausted, it will not be reprinted.

### USPHS Courses Begin in February

The Training Program of the Robert A. Taft Sanitary Engineering will present the courses, "Microbiological and Chemical Examination of Milk and Dairy Products," Feb. 1–5, and "Laboratory Methods for Prevention and Control of Food-Borne Disease," Feb. 8–12.

The first course is offered for professional personnel with a background in sanitary bacteriology in responsible positions in state, municipal, and other laboratories engaged in milk analyses and dairy products examination. Considered in the course are the theory, laboratory techniques, and interpretation of tests used in evaluating the sanitary quality of milk and dairy products, with practice in the special procedures used in detection of bac-

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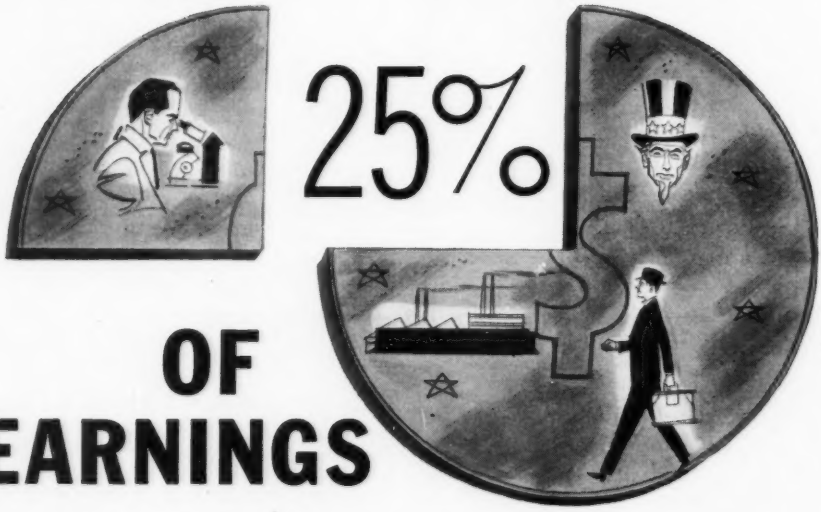

Research—the yardstick by which we prepare for the future—is of first importance at Diversey. Last year our expenditures for Research and Product Development amounted to about 25c of every dollar of pre-tax earnings.

Much of this effort is aimed at applying electronics and atomic physics to Dairy Sanitation. Advanced research like this has led to improved techniques for evaluating results of C-I-P cleaning, for instance.

In new-product development, an example of Diversey's many contributions is a new non-foaming conveyor chain lubricant—DICOLUBE SL. This lubricant provides cleaner, more smoothly operating chains and eliminates the problem of slippery, hazardous floors along conveyor lines.

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terial groups or of special significance in milk quality control.

The second course presents advanced technical training for laboratory and supervisory personnel concerned with examination of foods for bacterial or chemical contaminants. Emphasis is given to methods, standards, and procedures applicable to a food sanitation program. Laboratory relations to inspections, grading, and licensing of food establishments are considered.

Applications should be addressed to the Chief, Training Program, Robert A. Taft Sanitary Engineering Center, 4676 Columbia Parkway, Cincinnati 26, Ohio, or to a PHS Regional Office Director.

### U. S. Scientists Tour U.S.S.R.

The United States recently sent an Animal Husbandry Team to the U.S.S.R. as a part of the Cultural Exchange Program to have a look at the Soviet livestock industry and the research and educational programs relating to animal husbandry. This team was a counterpart to a U.S.-S.R. Animal Husbandry Team that visited the United States in the summer of 1958.

R. E. Hodgson

The team consisted of L. E. Hanson, University of Minnesota; Dr. E. E. Heizer, University of Wisconsin; A. V. Nalbandov, University of Illinois; A. H. Tillman, Oklahoma State University; W. Keller; C. E. Terrill; and R. E. Hodgson (Chairman), U.S.D.A. The team was in the U.S.S.R. from August 5 to September 7, 1959. During this time visits were made with animal husbandry research and educational specialists at the Ministry of Agriculture, agricultural institutes, and animal husbandry experiment stations. Visits were also made to some 15 State and collective farms. The tour included the areas in and around the cities of Moscow, Leningrad, Riga, Kiev, Kharkov, Askanaya Nova, Semferopol, Stavropol, Alma Ata and Rubtrovsk which included the important agricultural and livestock growing regions of the U.S.S.R.

The team members were impressed with the progress that the Soviet Union is making in the improvement in the production of cattle, sheep, swine and poultry and their products. A report of the results of this study will be forthcoming and made available to the public at a later date.

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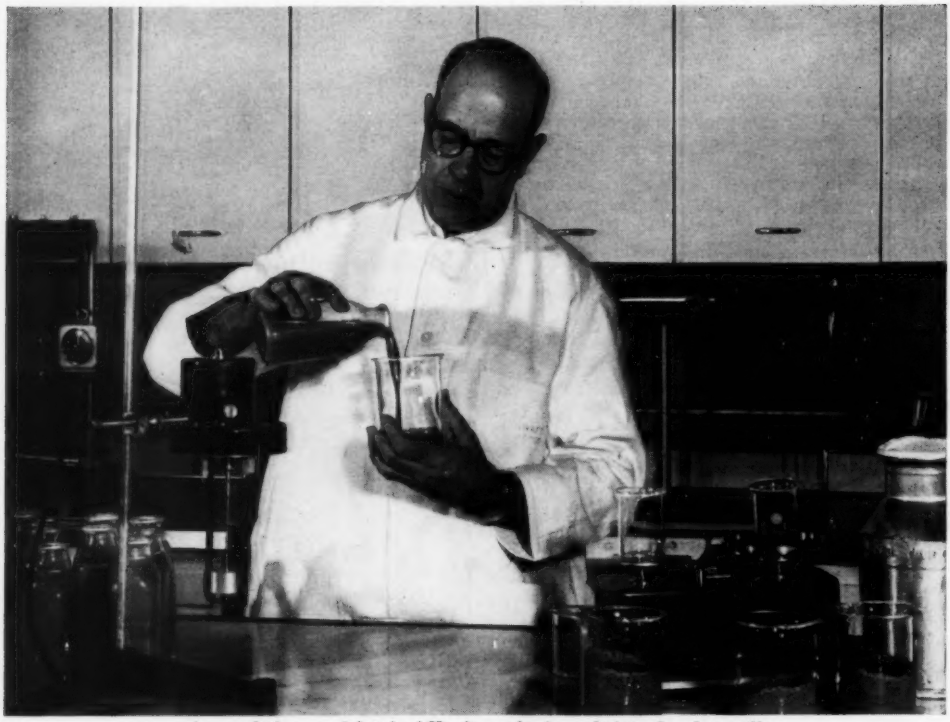

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## BUSINESS AND INDUSTRY NEWS

### Borden's News

The Borden Company has been named winner of the Public Relations News Annual Achievement Award for 1959, according to DENNY GRISWOLD, editor and publisher of the publication.

H. W. COMFORT, president of Borden's, accepted the award citation from Mrs. Griswold at a special luncheon in New York City. The award was made in recognition of the public relations program conducted by Borden's on the occasion of its recent centennial. It was one of 10 awards announced by "Public Relations News," which selected winners from more than 400 programs and activities the publication had reviewed during 1958.

Mr. Comfort, accepting the award, said, "No company can exist without the good will of its publics: stockholders, employees, suppliers, customers, and potential customers. To build and maintain this good will is a responsibility of everyone within an organization . . ."

MILTON FAIRMAN, the company's public relations director, served as secretary of the centennial policy committee and chairman of the centennial project committee.

---

IRVING GAMBERT has been appointed as a sales representative for malted milk in the New York metropolitan area. Mr. Gambert will handle sales to fountains and fountain supply houses. He has had many years of experience in the confectionery field as a broker and salesman.

### Milk Industry Foundation

G. H. HOOD, JR., vice president and treasurer of H. P. Hood and Sons, Boston, Mass., was elected president of the Milk Industry Foundation during the organization's 52nd annual convention held during October in Miami, Fla.

Mr. Hood, who has been with his family's dairy firm since 1920, has been vice president of M.I.F. for the past two years, and before that he was secretary. In Boston, he served as a director of the New England Council; a director of the Boston Chamber of Commerce; president of the Executives Club; president of Winchester Hospital; a trustee of Boston Lying-in Hospital; and a trustee of Andover-Newton Theological School and Wheaton College.

R. E. OSBORNE, president of Knudsen Creamery Co., Los Angeles, Calif., was elected vice-president of the Foundation. He has served on the board of directors for the past six years.

The new secretary is J. W. MARTIN, vice president of the Valley Bell Dairy, Charleston, W. Va. Mr. Martin has also been on the board. ALVIE CLAXTON, national director of trade re-


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4. Conduct research on new ideas suggested by you.

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October 26, 1959

Dr. X. X. XXXXXXXX, Professor
Department of Dairy Manufacturing
State University
Capital City, State

Dear Dr. XXXXXXXX:

The CP UltraTherm Infusion Heater is a versatile direct contact steam heater which is unequaled in efficiency and rapidity of heating the product. It is unique among high performance heaters in that it can utilize low temperature steam only a few degrees above the final product temperature. This, of course, is an advantage where the destabilizing effects of heat are to be minimized.

The UltraTherm is currently being used as a preheater, as the heater on Vac-Heat, as the final heater on UHT pasteurizers and many other applications in dairy and food processing.

Standard models are designed for heating to 285° F., but models for temperatures in excess of 300°F. are available on order.

A laboratory model suitable for research and pilot plant use is also available. It can handle any flow rate up to 4,000 lbs/hr., heating in any range to 300°F.

If you have further interest in the CP UltraTherm Infusion Heater or wish to consider a laboratory model for next semester's research projects, please write me or our branch which serves you.

Sincerely yours,

H. L. Mitten, Jr.

H. L. Mitten, Jr.
Director of Technical Sales

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lations for Beatrice Foods Co., Chicago, has been re-elected treasurer.

Two Canadians were elected to the board. They are GORDON SILVERWOOD, president of Silverwood Dairies, Ltd., London, Ont., and W. K. HOGG, president of the Guarantee Pure Milk Co., Ltd., Montreal, Que.

## DAIRY TECHNOLOGY SOCIETIES

**Atlanta**—CHARLES FRENCH, Agricultural Economics Department, Purdue University, was the main speaker at the October meeting of the society. His topic was "Getting the Right Perspective on Costs." New officers this year are NORMAN KOHL, president; STEPHEN SPECK, vice president; JAMES LITTLE, treasurer; and JOHN SHEURING, secretary.

**Central Michigan**—Dairy processing costs was the subject of ALVIN RIPPEN, agricultural marketing specialist from the Department of Agricultural Engineering, Michigan State University, at the October meeting.

**Detroit**—RICHARD KEPPELER, Michigan State University, spoke at the October meeting of the society. His topic was "Milk Meters."

**Metropolitan**—HANSON BLATZ, director of radiation control, New York City Department of Health, was the main speaker at the October meeting. He spoke on the topic, "Radiation Exposure of the Population from all Sources."

**Minnesota**—The Minnesota Society heard S. T. COULTER, head of the Department of Dairy Industries, University of Minnesota, at its first meeting. His topic was "Impressions Gained at the World Dairy Congress in London, England, in June, 1959."

**North Carolina**—"A Program for Improving Management Training" was the topic for October. The speaker was H. A. HOMME, dairy marketing specialist, North Carolina State College.

**Ohio**—MAX GREGORY, dairy extension specialist at Ohio State University, spoke at the October meetings of Ohio's four Dairy Technology Societies—Maumee Valley, Northeastern Ohio, Central Ohio, and Cincinnati. His topic was "Dairy Plant Efficiency."

**Philadelphia**—W. E. PETERSON, Dairy Husbandry Department, University of Minnesota, was the speaker at the October meeting. His topic was "Immunization through Milk Supply."

**Tri-Cities**—"Defects and Faults in Market Milk By-Products," was the topic featured at the October meeting. Main speaker was G. M. TROUT, Dairy Department, Michigan State University.

**Tri-State**—The October meeting featured a talk, "Our Changing Population and the Dairy Industry," by NORMAN MYRICK. Mr. Myrick is editor of the "American Milk Review" and "Milk Plant Monthly."

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IOSAN removes and prevents milkstone buildup. It simplifies sanitation because it is both a powerful cleaner and sanitizer. Reduces bacteria counts to consistent lows. Helps prevent mastitis.

IOSAN is the original patented "Tamed Iodine"® Germicidal Cleaner. Used daily by tens of thousands of milk producers. Especially effective for Bulk Tanks. For a free demonstration contact your regular supplier or Lazarus Laboratories Inc., Div. West Chemical Products Inc., 42-16 West St., Long Island City 1, N. Y.

**Utah**—JACK TRUNNEL, Dean of the College of Family Living, Brigham Young University, was the main speaker at the October meeting. His topic was "Carcinogens and Dairy Waxes."

**Western Michigan** — The October meeting featured a talk by L. G. HARMON, Dairy Department, Michigan State University. His topic was "Culture Quality."

# From the Secretary's Desk

### Maintaining an Effective Operating Staff

Recently I noted in the house organ of a division operating three sizable companies, the photographs and titles of the 36 key men in these companies. They were arranged in the form of an organization chart. Certainly, here is one organization that is giving consistent study and planning to maintaining an effective operative staff.

We find such key positions as follows: Vice President; General Manager of Marketing; Marketing Research Director; Sales Promotion and Advertising Director; General Manager Sales; General Manager of Operations; General Plant Manager; Chief Engineer; Industrial Engineer; Planning Director; Economist; Purchasing Director; Transportation Director; Milk Supply Manager; Quality Control Director; Public Relations Director; Personnel Director; Comptroller; Secretary-Treasurer. Because of the number of operations involved, some of these positions appear more than once.

I was impresed by the over-all youth of the organization, judging by the pictures. It would be interesting to have beside each picture the years of service with the company and the number of years to go prior to retirement at 65. Of course, the companies have this information.

It has seemed to me that many of the companies in the dairy industry have done considerable promiscuous employing, particularly of college graduates, without giving much thought as to where they may be needed or when they may be needed in key positions. Someone retires or dies or resigns and then there is apt to be confusion in filling the position instead of looking at an organization chart, such as I have mentioned, and then moving personnel about in an orderly manner.

Such an organization chart with photographs, titles and years of service for personnel can also be of inestimable interest and value to the young man considering employment in a company and in giving him some idea as to what may be expected in the way of promotions if he is successful. The relative youth of a live organization should help to instill patience in young men and make it clear that they must expect a number of years to pass before their pictures will appear on one of the top lines of the organization chart.

H. F. JUDKINS

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# THE FREEZING POINT OF MILK. A REVIEW[1]

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## SUMMARY

The literature clearly shows that the freezing point of milk varies within relatively narrow limits. Some of the variations that do occur have been attributed to seasonal effects, feed, water intake, time of day (i.e., morning versus evening milk), breed of cow, and method of handling samples. In handling milk samples, it has been reported that storing them at low temperatures, or freezing them, may raise the freezing point. Some analysts claim that heat treatment affects the freezing point slightly, whereas other workers have not observed any change. Vacuum treatment of milk has been reported to raise the freezing point slightly. Some of the apparent differences in freezing points reported by different workers may be due to differences in analytical techniques. Comparison of the freezing points reported by different analysts for identical samples reveals a need for more careful standardization of the method. It is believed that more attention should be given to the calibration of the thermal sensing element. It has been suggested that salt solutions or saturated aqueous solutions of stable compounds should be used as calibration standards instead of sucrose solutions. The necessity of standardizing the manner in which the thermometer is tapped has been emphasized. The ease and speed of determining freezing points with the recently designed cryoscopes, employing thermistors as thermal sensing elements, should increase the use of cryoscopes for routine analysis. The reported results show that the freezing point of milk is a useful index for detecting added water. However, the decision to classify a sample as watered must be based upon careful consideration of all of the available evidence. One should not rely on the conclusions of others without examining their data. The published evidence indicates that the present official standard in this country should be re-examined. It has been suggested that the adoption of a minimum freezing point standard might overcome the limitations of using an assumed average as a standard.

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In the past 10 yr., interest in the freezing point of milk has been stimulated by the publication of a number of papers concerning cryoscopic methods and standards. The results reported in these papers were believed by some to contradict previously published results; whereas, actually, most of the recent results could have been predicted on the basis of earlier literature. As is too often the case, interpretations or conclusions are handed down from generation to generation, but the facts on which they were based are overlooked or forgotten. An attempt will be made in this review to re-examine the conclusions in the light of the published facts.

### THEORETICAL CONSIDERATIONS

The freezing point of cow's milk is the least variable property of milk and, consequently, it is of interest from both a practical and theoretical standpoint. Its practical value is that it provides the most accurate means of detecting added water. It is of theoretical interest because of the physiological questions which it raises. For example, by what mechanism is the freezing point held within such narrow limits? The classical answer is that milk is in osmotic equilibrium with

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[1] Reprints available for 90 days after publication of this paper. For prices see page 1434 of the August issue.

the blood. (Incidentally, the freezing point is an indirect measure of the osmotic pressure.) The findings of van der Laan (100), Kern (54), and Koenig (58) support this answer. However, it does not explain the reason for the narrow range in osmotic pressure of the blood.

The osmotic pressure and, hence, the freezing point of milk or of any aqueous system is dependent on the concentration of water-soluble constituents. The addition of a solute to any solvent depresses the freezing point. The mathematical relationship between the freezing point depression and concentration of solute was worked out by Raoult (75) and is expressed in the following formula: $T_f = K_f m$, where $T_f$ is the freezing point depression, $K_f$ the molal depression constant, and $m$ the molal concentration of the solute. The molal depression constant differs for each solvent. In water, it is 1.86° C. As Raoult pointed out, this exact relationship is valid only for dilute solutions of undissociated solutes.

According to Cole and coworkers (21, 22), lactose and chlorides account for almost 75% of the total freezing point depression. They observed a slight difference between Jersey and Friesian milk. Their results indicate that over 50% of the depression is due to lactose and about 25% is due to chlorides. The fat and insoluble proteins do not have any measurable effect. Dharmarajan *et al.* (30) claimed that lactose and chlorides accounted for about 77% of the freezing point depression of cows' milk and about 72% for buffalo milk.

Pinkerton and Peters (70, 73) also studied the contribution of lactose to the freezing point depression and concluded that it was the most important factor. Rees (76), in a study of the variation of freezing points, stated that changes in the concentration of the nonchloride ash fraction were the primary cause of variations. The principal variable in this fraction was believed to be the soluble acid phosphates.

Numerous workers (14, 21, 22, 23, 50, 70, 72, 73) have pointed out the complementary nature of the concentration of lactose and chloride. In general, as the concentration of lactose decreases, the chloride concentration increases, and vice versa. The fact that the freezing point is less variable than other properties of milk has been attributed at least in part to this complementary relationship. However, Aschaffenburg and Rowland (6) noted a case where this complementary relationship did not exist. They found that the lactose-chloride ratio of morning versus evening milk did not change, even though there was a change in the freezing point. They stated that evening milk appeared to be naturally watered morning milk.

## CRYOSCOPIC METHODS

In 1921, Hortvet (45) published a new method for determining the freezing point because he felt that earlier methods were not sufficiently standardized. Since there is no published proof that earlier methods gave data comparable to Hortvet's data, this review will deal only with the literature pertaining to the Hortvet method, or methods reported to give results comparable to this method.

Hortvet recognized the empirical nature of cryoscopic procedures and, therefore, he emphasized the need for using standardized equipment and technique. To

minimize variations between analysts, he gave a detailed description of the apparatus and procedure. As an additional device to compensate for variability between analysts, his procedure involves the measurement of the difference between the freezing point of a standard solution (i.e., 7% sucrose) and the freezing point of milk. Theoretically, any systematic errors should be reflected in both observed values and should offset each other. This feature of the Hortvet technique places a special emphasis on the calibration procedure that is apparently not recognized by all analysts. Of course, the calibration procedure will compensate for systematic errors only when variations in technique have exactly the same effect on the observed results for both the standard solution and milk. Hortvet's choice of sucrose solutions as calibration standards may have been unfortunate, since the freezing characteristics of sucrose solutions and milk are different, according to Shipe (83). This observation suggests that variations in technique may not have exactly the same effect on the standards as on the milk and, hence, compensating effects are reduced. Furthermore, it is difficult to obtain reproducible results with sucrose solutions and they readily undergo microbial decomposition.

To overcome the difficulties encountered with sucrose calibrations, Aschaffenburg and coworkers (4, 5, 7) have recommended that the thermometers should be calibrated by a recognized testing institution such as the British National Physical Laboratory. Sutton and Markland (95, 96) have questioned the wisdom of such a procedure and have presented data indicating that the sucrose calibrations give better results. Some analysts have reported that salt solutions give more reproducible results than sucrose solutions. Shipe (84) has suggested the use of stable compounds whose solubilities in water are such that saturated aqueous solutions of these compounds would have freezing points in the desired range. It is this author's opinion that Hortvet's idea of determining the difference between the freezing point of a standard and milk is basically sound.

Attempts have been made to apply a correction factor to the observed Hortvet freezing point, to compensate for the deviation from the true freezing point. Monier-Williams (68) and Stubbs (89–92) have reported on their efforts to determine the magnitude of this deviation. Elsdon and Walker (37) have given an excellent review of the factors involved. However, from a practical viewpoint it is not necessary to determine the true freezing point. The key problem lies in obtaining reproducible results between samples and analysts.

Horvet (45) assumed that reproducible results could be obtained by his method. However, he did not report any evidence to prove this assumption. It is generally assumed that a given analyst can obtain reproducible results. On the other hand, there is reason to question the assumption that different analysts will obtain comparable results. A report by Shipe, Dahlberg, and Herrington (85) revealed considerable differences between the observed results for nine collaborating laboratories. For one series of seven samples, there was a maximum variation of 0.0275° C. between the average freezing point values. However, in most cases, a given laboratory deviated from the average for each sample by

about the same amount. This indicated that each analyst was able to reproduce his own work, but his values were not necessarily comparable to those of other analysts. A collaborative study by Robertson (79), involving 19 laboratories, revealed similar results. The collaborators were supplied with three milk samples, two of which were identical; the third sample contained 1.5% added water. With the exception of five laboratories, they were able to correctly differentiate between samples. However, there were considerable variations in the observed freezing point values. Additional evidence of the variations between laboratories is reported in a collaborative study by Shipe (83).

In contrast to the variable results reported, Elsdon and Stubbs (35) reported good agreement between six laboratories on one sample of milk. Using a procedure prescribed by Aschaffenburg and King (5), seven laboratories were able to obtain comparable results. In this case, the analysts used an 8.5% sucrose solution and, therefore, knew the expected results in advance. Nevertheless, these two reports indicate that good agreement can be obtained between analysts with the Hortvet method. However, numerous workers have observed causes for lack of reproducibility and have in some cases suggested modifications.

Elsdon and Stubbs (33) observed that the thermometer calibrations varied with the storage temperature and age of thermometers. Jones (52) observed that leaving the starter tube in the sample during the freezing affected the observed freezing point of water. Monier-Williams (68) suggested that the alcohol be removed from the tube surrounding the sample tube at the time of seeding, so as to minimize heat transfer during the freezing process. Stubbs (91) made a similar recommendation. Stubbs and Elsdon (94) made specific recommendations for standardizing thermometers.

Temple (99) designed a modified cryoscope in which a mechanically refrigerated bath was substituted for Hortvet's ether-cooling system. This cryoscope was easier and it permitted better control of the bath temperature. Temple's cryoscope has been used extensively in England.

Sutton and coworkers (97) have studied some of the potential sources of errors in the Hortvet procedure. They pointed out that due to the slow adjustment of glass to changes in temperature the zero point of the thermometer might change as much as 0.005° C. during a day. They also noted that the dimensions of the freezing tube and thermometer bulk were critical. They stated that it is essential to limit the amount of stirring after seeding. They suggest modifying the Hortvet apparatus so that the alcohol surrounding the sample tube can be removed at the time of seeding.

Shipe, Dahlberg, and Herrington (85) have designed a modified cryoscope which employs a mechanically refrigerated bath. Their cryoscope also has mechanical devices for stirring the sample and for tapping the thermometer. They observed that mechanical tapping markedly reduced variations between analysts. This observation has been confirmed by Dubin (32). Green (40) has designed a cryoscope with a mechanical stirrer and tapper. He has also observed better results using these mechanical features. Incidentally, Monier-Williams (67) in 1915 had used a mechanical tapping device.

Gaballah (39) designed a cryoscope with four freezing tubes. His apparatus is mechanically refrigerated and the sample is stirred mechanically. He claimed that four determinations could be made in 15 min. Knowles (57) modified an electric water cooler to provide a bath for a cryoscope.

Bowman, Trantham, and Caulfield (16) developed a cryoscope, using a thermistor as the thermal sensing element in place of a mercury in glass thermometer. The American Instrument Company, Inc.[1] and Fiske Associates, Inc.[2] have manufactured cryoscopes which use thermistors. Results obtained with the Fiske cryoscopes have been reported by Hileman and Wiggin (42) and Shipe (82, 83). The results indicate that determinations can be made easily and rapidly (20 to 30 per hour) with this cryoscope. Shipe's data indicate that it is possible to obtain results with the Fiske that are comparable to the Hortvet. However, the results indicate that a more standardized procedure is necessary to reduce variations between analysts.

The British Standards Institution (17) has just published a revised set of directions and specifications for the Hortvet method. This publication contains a very detailed description of the apparatus and the procedure which overcomes some of the ambiguities of Hortvet's directions. However, the new procedure for tapping the thermometer seems to be unnecessarily complicated. This procedure was first recommended by Aschaffenburg and King (5). Shipe (84), by using a mechanical tapper which minimizes the human factor, obtained the same results as with the more complicated British method.

## FREEZING POINTS OF COW'S MILK

In 1923, the Association of Official Agricultural Chemists (48) officially adopted the Hortvet cryoscopic method. In connection with the adoption of the method, they recommended that −0.550° C. be considered the average freezing point of normal milk, with the allowance of a 3% tolerance. These recommendations were based on the observations of Hortvet (45, 46) and a collaborative study conducted by Bailey (12). Libby, McNeill, and Libby, Chicago and Morrison, Illinois, the Connecticut Agricultural Experiment Station, and the Minnesota Agricultural Experiment Station participated in this study. The results are shown in Tables 1 and 2.

As will be noted in Table 1, the average freezing points of 179 individual cow samples and 61 herd samples were −0.545 and −0.544° C., respectively. It should be noted that there are appreciable differences between the averages obtained by the three laboratories. For example, the herd average for Libby, McNeill, and Libby laboratory was −0.542°, whereas the corresponding value for the Minnesota samples was −0.551°. This difference is equivalent to about 1.5% added water. This difference between laboratories might be interpreted as indicating that the milk supply was different in the two areas, or that the tech-

[1] American Instrument Co., Inc., Silver Spring, Maryland.
[2] Fiske Associates, Inc., Hathorne, Massachusetts.

TABLE 1
Summary of freezing point data reported by Bailey (12)

| Laboratory | No. of samples | Maximum ° C. | Minimum ° C. | Average ° C. |
|---|---|---|---|---|
| | | Individual cow samples | | |
| Minnesota | 77 | −0.562 | −0.534 | −0.547 |
| Libby, McNeill, & Libby | 27 | −0.563 | −0.532 | −0.546 |
| Connecticut | 75 | −0.566 | −0.530 | −0.543 |
| | 179 | −0.566 | −0.530 | −0.545 |
| | | Herd samples | | |
| Minnesota | 15 | −0.562 | −0.545 | −0.551 |
| Libby, McNeill, & Libby | 37 | −0.553 | −0.530 | −0.542 |
| Connecticut | 9 | −0.560 | −0.535 | −0.544 |
| | 61 | −0.562 | −0.530 | −0.544 |

TABLE 2
Dates of collection of freezing-point samples as reported by Hortvet (45) and Bailey (12)

| Laboratory | No. of samples | Dates collected |
|---|---|---|
| | Individual cow samples | |
| Minnesota | 15 | May |
| | 25 | June |
| | 1 | August |
| | 19 | September |
| | 17 | Dates not reported |
| Libby, McNeill, & Libby | 27 | September (14 to 23) |
| Connecticut | 73 | April 5 to May 26 |
| | 2 | July 7 |
| | Herd samples | |
| Minnesota | 15 | Dates not reported |
| Libby, McNeill, & Libby | 2 | March 29 |
| | 3 | April 1 |
| | 5 | June |
| | 4 | July |
| | 2 | August |
| | 21 | September |
| Connecticut | 9 | April 13 to May 2 |

niques used by the different laboratories were different, or both. No attempt had been made to check the reproducibility between laboratories. Of course, the differences may have been due to the limited number of samples analyzed.

In addition to the number of samples being limited, the period of collections was limited. As is shown in Table 2, there were no samples reported as having been collected during October, November, December, January, or February. This failure to collect samples in the winter months apparently was due to the assumption that the season of the year has no effect on the freezing point.

The fact that Bailey's collaborative study revealed differences between the freezing point of morning and evening milk generally has been overlooked. Of the samples reported, 49 were identified as evening samples and 44 as morning samples, with average freezing points of −0.542 and 0.555° C., respectively.

It appears that the results and limitations of this initial work with the Hortvet method have received less attention than the following statement of Hortvet (45)

that: "Milk, freshly drawn, from any variety of cow whether of high or low breed, from whatever region of the country, from animals in stable, or in pasture, whether poorly or substantially fed, whether drawn during a period near or remote from parturition, in winter or in summer, morning or evening, or whether the yield be scant or abundant, has a freezing point which varies but little around −0.550° C., although under various influences the chemical composition changes in enormous proportions." This statement has been accepted by many as meaning that the freezing point is a constant, in spite of the qualifying phrase, "which varies but little around −0.550° C." The statement does imply that the freezing point is independent of environmental factors. This implication has been widely accepted, although their data do not substantiate it. In fact, there is considerable evidence to prove that environmental factors do influence the freezing point.

Buchanan and Lowman (19, 20) observed changes in freezing points with the seasons. They concluded that these changes were due primarily to changes in feed. However, they stated that meteorological conditions may have been a contributing factor. Regan and Richardson (78) observed that changes in environmental temperature affected the freezing point. Aschaffenburg and Veinoglou (9) suggested that differences in environmental temperature were a contributing factor to the seasonal variations that they observed.

Koenig (58) observed that cows on a semistarvation diet of poor-quality potato pulp gave milk with abnormally high freezing points. Rees (77) attributed most of the seasonal variations to differences in feed. Bagnall and Smith (11) observed that milk from cows which had recently been changed from stall feeding to grazing had abnormally high freezing points. Gaballah (39) also observed a change in the spring, with the shift to pasture feeding.

Pinkerton and Peters (1) observed that when a herd was shifted from a high- to a low-carbohydrate diet the freezing point of the milk was raised and the solids-not-fat was lowered.

Shipe, Dahlberg, and Herrington (86) observed differences in both the averages and ranges of freezing points of milk from five cows on three different rations. They observed similar results in another experiment involving nine cows on two rations. On the other hand, Kleyn *et al.* (56) did not find any significant difference between the average daily freezing points for milk produced on different rations. However, they did observe differences in the relationship between the freezing points of evening and morning milk for the different rations.

As was pointed out previously, Hortvet (45) assumed that the freezing point was independent of the breed of cows. However, there is evidence which raises questions about this assumption. Aschaffenburg and Veinoglou (9) noted that the freezing points of Guernsey milk were slightly higher than for Shorthorns. Krienke and Arrington (59) reported differences between Jerseys and Holsteins. Shipe, Dahlberg, and Herrington (86) reported differences between Brown Swiss and Holsteins. Bryant and Briggs (18) reported differences between Friesians and Guernseys. Peters, Mulay, and Shrode (70) reported differences between Holsteins and Jerseys. Sato *et al.* (81) determined the freezing point of milk

from five different breeds. They reported slight differences and pointed out that Ayrshire herds showed the highest average freezing point. Robertson (79) noted some differences between breeds in his national survey, but because of other variables it is difficult to evaluate the significance of differences in breeds. The amount of data on breed differences is insufficient to make any definite conclusions.

Notwithstanding Bailey's (12) observation that there were differences between the freezing point of morning and evening milk, it appears that some workers have assumed that there were no differences. This assumption was supported by the work of Stubbs and Elsdon (93), who reported an average freezing point of −0.545° C. for 440 morning samples and −0.544° C. for 550 evening samples. Kleyn and Shipe (55) found an average freezing point of −0.537° C. for 541 A.M. samples and −0.544° C. for 536 P.M. samples. Aschaffenburg and Temple (7) found that the average freezing point of morning milk was 0.002° C. higher than evening milk. In another study, Aschaffenburg and Veinoglou (9) found that the difference between morning and evening milk varied with the season. They found that in the winter the freezing point of evening milk was higher, whereas during the rest of the year the morning milk was higher. Shipe, Dahlberg, and Herrington (86) and Kleyn *et al.* (56) noted that the differences in freezing points between morning and evening samples varied with the ration. Kleyn also observed that the time interval between feeding and milking influenced these differences in freezing points.

Several workers (6, 43, 44, 49, 101) in England have shown extreme differences between morning and evening samples. These differences have been attributed to the fact that the cows had access to water only between the morning and evening milking. The morning milk appeared concentrated (i.e., had a low freezing point), whereas the evening milk appeared to be watered. The results of Aschaffenburg and Rowland (6) illustrate the type of differences obtained. On four different dates they found the freezing points of milk from a 32-cow herd to be −0.525, −0.528, −0.526, and −0.531° C. for evening milk; whereas, the corresponding values for morning samples were −0.555, −0.556, −0.560, and −0.566° C. This herd was kept in the barn from 4 P.M. to 9 A.M. and no water was available during this time. After the cows had been turned out to pasture, and had access to water both night and day, the freezing points were again determined on the mixed-herd milk. The values obtained on two different dates were −0.553 and −0.542° C. for the evening milk and −0.546 and −0.543° C. for morning milk.

It should be obvious from these findings on morning and evening differences that if one wishes to establish the authentic average for an individual cow or herd, it will be necessary to obtain samples of both morning and evening milkings.

A number of studies has been made to determine the effect of handling or treatment of milk on its freezing point. The effect of developed acidity was recognized by the early analysts. Bailey (13) studied this effect and noted an average freezing point lowering of about 0.003° C. for each 0.01% increase in acidity. Lythgoe (63) has suggested the use of a series of adjustment factors to correct for developed acidity. The official manual (10) states that "If the

titratable acidity, 15.4, exceeds 0.18%, results may underestimate the actual amount of added water in the sample." To prevent the development of acidity, Aschaffenburg, Temple, and Veinoglou (8) added 0.01% mercuric chloride. They applied a correction of 0.004° C. to those samples to which preservative had been added.

Rao, Dastur, and Dharmarajan (74) tested 11 preservatives and recommended the use of 0.075% mercuric chloride, for which they recommended a correction factor of 0.020° C. They reported an average difference of 0.019 ± 0.0082° C. between the original freezing point and the freezing point of milk, after the preservative had been added and the samples kept 1 wk.

While the development of acidity with storage has been recognized as a potential source of error, there is another, less-recognized effect of aging. Pinkerton and Peters (72) presented data showing the effect of storing ten samples of milk at 0° C. for 48 hr. In eight samples, the freezing point had changed and in all eight it had risen. The increases in freezing points ranged from −0.001 to 0.004° C. and were accompanied by decreases in conductivity. The author of this review has noted similar shifts in the freezing point when milk was stored for 24 to 48 hr. at 5° C. Where there were shifts, they were always up, indicating a decrease in the concentration of solutes. It is assumed that this is due, at least in part, to a decrease in the solubility of salts.

Freezing milk also has the effect of raising the freezing point. Shipe (84) reported results on two sets of samples which had been frozen and stored for 24 and 48 hr. He also noted that if one refreezes a milk sample, the freezing point of the second determination may be higher. A change in the freezing point was not observed in all cases, but in those where it did occur the freezing point of the replicate was always higher. On the basis of these observations, the author advises against refreezing an aliquot of milk as a duplicate.

A limited number of experiments have been conducted to determine the effect of heat on the freezing point. Monier-Williams (67) stated that heating for 20 min. at 60° C. raised the freezing point about 0.002° C. Elsdon and Stubbs (34) noted a slight tendency for the freezing point to be raised as a result of pasteurization or sterilization. However, the amount of change was small, and in some cases there was no change. Dharmarajan, Rao, and Dastur (28) reported that pasteurization did not affect the freezing point, but that boiling or simmering lowered it. This lowering may have been due to evaporation of water. Staub and Krahenbuhl (88) observed no change after pasteurizing with the holder method or after boiling for 10 min. under reflux. Pinkerton and Peters (72) reported that heating the milk to 161° F. for 16 sec. raised the freezing point and that heating to 169° F. for 16 sec. had a greater effect. They noted a corresponding decrease in the conductivity upon heating. Aschaffenburg *et al.* (3) reported that ultra-high-temperature treatment did not have any effect on the freezing point. Lazar and Henningson (61) observed that vacuum pasteurization raised the freezing point about 0.006° C. They attributed this shift to the removal of dissolved gases. Some of the changes noted by the other workers might be attributed to removal of dissolved gas. The lack of a detailed description of the

equipment used does not enable one to evaluate whether this could be an explanation for the apparent discrepancies. However, except for vacuum pasteurization, the changes observed by the various workers are so small that it is doubtful whether they have any practical significance.

The increasing use of vacuum treatment in processing milk does create a problem in interpreting freezing point data. However, except for the reference cited above there is no published information on this subject.

Doan (13) has shown that the freezing points of cream and skimmilk were the same as the original whole milk. However, they noted a relative sluggishness in the rate of rise of the mercury thread for the cream as compared with milk. Shipe, Roberts, and Blanton (87) have also observed differences in the apparent freezing rates for cream and milk. Their results suggest that the rate is related to viscosity. From a practical standpoint, the analyst should be sure to wait long enough after seeding viscous samples to insure that the maximum temperature has been reached.

Numerous papers have been published on the range and average freezing points. On the basis of the work and interpretations of Hortvet and Bailey (12, 45–48), the official average is −0.550° C. and the upper legal limit is −0.5335° C. in this country. The limitations of this work previously have been discussed. In a study involving 1,000 samples, Stubbs and Elsdon (93) in England obtained an average of −0.544° C., with a range from −0.529° to −0.563° C. Lampert (60) analyzed 20 samples of bulk milk and found an average value of −0.536° C., with a range of −0.529 to −0.540° C. On the basis of his findings, plus these of Hortvet, Bailey, and Stubbs and Elsdon, he recommended using −0.540° C. rather than −0.550° C. as the standard value.

Tankard and Bagnall (98), in a study of daily variations in the freezing point of evening milk over a four-day period, observed a range of −0.541 to −0.559° C. for 24 herd samples and −0.536 to −0.564° C. for 112 individual samples. Lythgoe (62) reported a range of −0.532 to −0.578° C., with a medium of −0.5535° C. for 73 authentic samples collected in Massachusetts during 1943-1944, and a range of −0.510 to −0.585° C. with a median of −0.5525° C. for 133 samples collected between 1920 and 1925. In a subsequent paper, Lythgoe (64) reported a range of −0.527 to −0.583° C. with a median of −0.548° C. for 148 individual cow samples collected between 1935 and 1946. Arrington and Krienke (2) reported a range of from −0.529 to −0.564° C. for 64 authentic samples.

Dharmarajan *et al.* (30), in India, reported an average freezing point of −0.548° C. for 535 herd and 307 individual samples. In another report on the composition of milk taken from different farms throughout India, Dastur *et al.* (27) reported an average of −0.545° C. for 202 samples.

Gaballah (39) collected 1,086 samples of individual and herd milks during 1950 through 1953 in Wisconsin. He reported an average of −0.546° C., with a range from −0.531 to −0.562° C. Sato *et al.* (81) reported an average of −0.546° C., with a range of from −0.533 to −0.560° C. for 59 authentic herd and individual cow samples collected in Ohio. Kleyn and Shipe (55) determined the freezing

point of 541 samples of evening milk from individual cows in the Cornell herd and reported an average of −0.541° C. with a range from −0.513 to −0.571° C.

Robertson (79) conducted a survey of the freezing point of milk from 16 areas in the United States and one in Canada. The reported average freezing point was −0.543° C. for 1,627 samples. The average freezing points reported from the separate areas ranged from −0.528 to −0.549° C. However, when the laboratories from the different areas determined the freezing point of two "standard milks," the differences between the reported values for the same samples of milk were of about the same order of magnitude as the differences between the "area milks." Therefore, it would seem that the analysts contributed as much to the variations in reported results as did the cows.

A number of workers have studied the freezing point of bulk milk which provides a basis for estimating the extent of adulteration. Aschaffenburg, Temple, and Veinoglou (8), in England, tested 4,000 samples of milk representing mostly individual producer samples. They stated that about six samples per hundred were found to contain water. From the distribution of freezing points, they concluded that it was unwise to calculate per cent of added water from an assumed mean of −0.550° C. They also noted definite seasonal effects—the freezing points were lower in May than during January to April.

Paley and Tzall (69), in New York City, determined the freezing points of 1,450 fluid milk samples obtained from milk dealers, their representatives, and duplicates of inspectors' samples. Only 40% of the samples showed freezing points within the 3% tolerance allowed by the A.O.A.C. Thirty-three per cent of the samples had freezing points between −0.535 and −0.530° C. On the basis of their data, they suggested that a study should be conducted to determine if the official value of −0.550° C. was valid for our present milk supply.

MacDonald (65), in England, collected 480 samples of milk from 3,000-gal. bulk lots over a period of a year and found that the freezing points varied from −0.542 to −0.548° C., with an average of −0.544° C. In a more extensive study between 1942 and 1947, he determined the freezing point of 9,543 samples of bulk milk and reported an average freezing point of −0.543° C. It should be noted that he did not report any freezing points below −0.550° C. in either of his studies.

Rees (76) reported a range of values from −0.514 to −0.570° C. for 155 samples of bulk market milk from one area of Tasmania, and a range of from −0.506 to −0.558° C. for 158 samples from another area.

Dahlberg, Adams, and Held (26) determined the freezing point of 135 samples of commercial pasteurized milk from eight cities located in different areas of the United States. These samples represented about eight million quarts of milk. The average freezing point of these samples was −0.540° C. The milk from two plants was definitely shown to be watered and excluding the results from these plants the average was found to be −0.541° C. Only 23 samples had freezing points of −0.550° C. or lower. They concluded that the distribution of freezing points cast some doubt on the A.O.A.C. standard of −0.550° C.

On the basis of lactometer readings and fat tests, Yongue (102) reported that 3,460 out of 28,368 samples of fluid milk were classified as suspected. The freezing points of 1,057 of these suspected samples were determined. Of these samples, 21.5% were found to have freezing points above −0.520° C. and 16.5% had freezing points between −0.520 and −0.529° C., inclusive.

Sato *et al.* (81) reported an average of −0.538° C., with a range from −0.450 to −0.550° C. for 108 samples of producers' milk, and an average of −0.530° C., with a range from −0.437 to −0.549° C. for 53 samples collected from retail outlets. They stated that 94% of the producers' samples would have been classified as unadulterated if −0.530° C. was used as the legal limit; whereas, only 87% would have been so classified if −0.534° C. was used. The retail samples showed 72 and 57%, respectively, by these two standards.

### FREEZING POINTS OF THE MILK OF OTHER MAMMALS

There have been a limited number of studies on the freezing points of milk from other mammals. The most extensive studies have been made on buffalo milk. Dharmarajan *et al.* (30) reviewed the literature and reported that they had found an average of −0.552° C. for 193 individual and 271 herd samples of Indian buffalo milk. In another study of Indian buffalo milk, Dastur *et al.* (27) reported an average of −0.544° C. for 37 herd samples. Dharmarajan *et al.* (29) reported an average of −0.579° C. for goats' milk and −0.587° C. for ewes' milk. Kern (53), in Israel, reported an average of −0.565° C. for ewes' milk. Jax (51), in a study of the properties of sows' milk between the 2nd and 10th wk. of lactation, reported a range of freezing points from −0.528 to −0.683° C. Anselmi and Caló (1) obtained seven samples of elephants' milk during the 1st and 2nd wk. after parturition, and reported a range from −0.548 to −0.554° C. Miller and Ellis (66) determined the freezing point of 260 samples of milk from 215 healthy women and reported a range from −0.531 to −0.586° C., with a mean of −0.564 ° C.

### FREEZING POINTS AS AN INDEX OF ADDED WATER

While the freezing points of the commercial samples provide an indication of the magnitude of the adulteration problem, the variations in the reported freezing points of authentic samples provide an indication of the problem of establishing appropriate standards.

In the past, Winter's table, as cited by Henderson and Meston (41), has been accepted by some as indicating the exact relationship between freezing points and percentage of added water. A literal interpretation of this table requires the assumptions that the freezing point of pure milk is constant and that the correct value is −0.550° C. Ample evidence has been cited to invalidate any assumption that the freezing point is constant. Furthermore, the evidence does not even support the assumption that −0.550° C. is the correct average. It should be pointed out that Winter's table was prepared before the development of the Hortvet method and that there is no published evidence to prove that Winter's and Hortvet's methods gave identical results.

One other criticism of Winter's table is that the calculations do not include any factor to compensate for the solids content of milk. His formula, which has been adopted by the A.O.A.C. (10), was:

$$\text{Per cent added water} = \frac{T-T'}{T}\,(100)$$

where $T$ is the average freezing point (i.e., −0.550° C.) and $T'$ is the observed freezing point. On the basis of their studies, Elsdon and Stubbs (36) have recommended the following formula:

$$\text{Per cent added water} = \frac{T-T'}{T}\,(100 - TS)$$

Bird and coworkers (15, 103) have shown the superiority of the Elsdon and Stubbs formula. To overcome the errors resulting from the use of too low an average, and from ignoring the solids content, Freeman (38) has recommended the following formula:

$$\text{Per cent added water} = \frac{(0.544 - T')\ 0.92}{0.544} \times 100$$

Regardless of the formula used, the problem lies in a choice of a value for the average freezing point. Obviously, the formula will be correct only if the proper value is chosen and in most cases this will not be known. Therefore, in most cases, the calculated percentage of added water represents only an approximation. To obtain a more reliable approximation, some regulatory agencies have adopted the practice of collecting authentic samples from the source of suspected samples.

Another means of verifying freezing-point results is to determine other properties of the suspected samples, such as the fat and solids content and the specific gravity. This procedure has been recommended by a number of workers (24, 62, 64, 79, 80). In some cases (24, 102), these tests are used for screening purposes, with the freezing point determination being used as a confirmatory test. Presumably, this practice has been followed because of the time consumed in running freezing points with the Hortvet apparatus. However, in view of the ease and speed of determining freezing points with the Fiske cryoscope, this test may be used more frequently for screening purposes. Since the freezing point test is a more accurate index of added water than other tests, it should be used as the test of first choice. In marginal cases, other tests, or comparison with authentic samples, should be made.

This brings us to the problem of establishing judicious freezing point limits. The British and Australians have been using −0.530° C. as an upper limit. The British Standards Institution (17) has recommended using an average of −0.540° C. for milk known to represent at least 200 gal., and an average of −0.530° C. for all other milks, in computing the percentage of added water. It recommends reporting this calculated value as "the minimum percentage of added water." This method of reporting calls attention to the fact that the calculated value is not necessarily the exact value. The use of different standards for large and small volumes of milk is at least partially justified, since abnormal

values from individual cows would be "diluted out" in bulk supplies. However, it is conceivable that a large herd could produce milk with an abnormal freezing point as a result of unusual environmental conditions. It is worth noting that the highest freezing point reported by Bailey (12) was the same (i.e., −0.530° C.) for both herd and individual cow samples.

Dahlberg (25) has suggested that −0.527° C. be fixed as the upper legal limit for the freezing point. [His choice of −0.527° C. was based on allowing 3% tolerance from the average of −0.543 ° C., obtained by Robertson (79) in his national survey.] Dahlberg points out that the laws regarding minimum standards for fat and solids serve as a precedent for this approach. Certainly, this approach would eliminate controversies about averages and the method of calculating added water. From the standpoint of fairness, it would be in the same category with the other minimum standards. In view of the normal variations, the fact that a sample meets minimum specifications does not necessarily indicate that the milk is free from added water. The British Standards Institution (17) also points this out in connection with its revised standard. The fact that a sample meets the minimum requirement would not prevent a regulatory agency from prosecuting if they had sufficient evidence of added water.

Robertson (79) has suggested the establishment of local standards as an alternative to a general standard. This alternative has merit if the freezing points vary consistently and significantly from one area to another. Of course, some of the apparent differences may be due to differences in analytical technique. This emphasizes the need to standardize the method. It would be unfortunate if a number of standards were established just to compensate for variations in the technique.

## CONCLUSIONS

The observations of Hortvet and Bailey, that the freezing points of milk vary within relatively narrow limits, and that the freezing points of morning and evening milk may be different, have been substantiated.

Hortvet's conclusions that the average freezing point is −0.550° C. and that a 3% tolerance calculated from this value represents a reasonable standard, have not been substantiated. His assumption that different analysts will be able invariably to reproduce one another's results has not been verified. In fact, the literature indicates that a lack of agreement between analysts may be a significant cause of apparent variations in the freezing point of milk. His assumption that environmental factors do not affect the freezing point has been disproved.

The results indicate that the basic features of the Hortvet method are sound. However, the results indicate a need for more careful standardization of the method. The design of cryoscopes using thermistors as the thermal sensing elements may give impetus to the use of cryoscopic techniques, because of the ease and speed of making analyses.

In spite of the variations in freezing points and techniques, freezing points do provide an excellent index of added water. More general recognition of the limitations of the method, and judicious interpretation of the results, should help to avoid misinterpretations of cryoscopic results.

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# CIRCULATION CLEANING. III. THE KINETICS OF A SIMPLE DETERGENT SYSTEM

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## SUMMARY

Cleaning of milk films by solutions of sodium hydroxide is shown to follow the kinetics of a first-order reaction. A molar velocity constant is calculated, and the role of solution temperature is evaluated in terms of its effect on the rate of the reaction. The reaction between solutions containing hydroxide ion and milk films exhibits a $Q_{10}$ of 1.6 under these conditions, and the effect of temperature on the reaction rate constant is linear, at least within the limits of 36 to 82° C. Application of kinetics to detergency permits temperature effects to be measured rather than estimated, and will allow studies of compatibility, incompatibility, or complementary interaction of detergent fractions, and selection of detergents best suited to particular requirements.

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Although it is generally recognized that in most cleaning regimes the concentration of the detergent exercises an effect on the rate of soil removal, there has been no precise evaluation of this effect. This has been due, in part, to variability in soil films, the lack of a suitable method for accurately determining the amount of soil removed under standardized conditions, and the fact that such determinations do not yield precise points. Too, many of the preliminary investigations that have been made utilized plants in commercial operation. Studies made under these conditions have been handicapped, in that the cleaning regime under investigation must accomplish satisfactory cleaning; the most the investigator could hope to establish from such studies would be the minimal conditions to accomplish cleaning. It is not a simple matter to evaluate variables properly under this restriction.

Because the reaction(s) between films of deposited soil and detergent fractions would demand that the two species come in contact, the cleaning mechanism can be considered as somewhat analogous to a bimolecular reaction, and should be amenable to treatment from the standpoint of chemical kinetics. If this were true, not only could detergents be selected to do a particular cleaning job with greater efficiency, but the effect of variables such as temperature might be measured rather than estimated. Various authorities have recommended temperatures ranging from 115-180° F. for cleaning solutions (1, 2, 3, 6), and some (7) have pointed out that a major advantage of circulation cleaning is that higher temperatures are permissible. This work was directed to testing the application of chemical kinetics to detergency, and establishing methods for more precisely evaluating detergent efficiency. The studies reported herein were limited to the removal of milk films by hydroxide ion.

### METHODS AND PROCEDURE

0.50-ml. aliquots of $P_{32}$-labeled milk were cooked on stainless steel test disc inserts machined to fit the inner periphery of a test section of 1½ o.d. stainless

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steel tubing (5). The amount of soil removed by particular cleaning regimes was followed by determining the decrease in the radioactivity of the test discs. Previous work has indicated that the radioactivity of films produced from in vitro labeled milk serves as a suitable index of both organic and inorganic milk residues (5).

The test apparatus used has been described elsewhere (4). It was modified slightly by coiling 10 ft. of ¾-in. o.d. copper tubing in the reservoir. This permitted temperature adjustment (by conducting cold water or steam through the coil) without dilution of the solution. The test section was on the pressure side (between the pump and the outlet) and possessed a positive slope of ¼ in/ft to insure flooded conditions. Solutions in 300-lb. lots were maintained at 46° C. (except as noted) and circulated at a Reynolds number of 550,000 (equivalent to 18.6 lb/sec or 29.4 ft/sec). These conditions of high turbulence minimized diffusion and concentration gradients of free hydroxide ion at the soiled surface, and assured a continuous supply of hydroxide ion at essentially constant concentration. The 300 lb. of solution contained 55, 165, 275, 550, and 825 g. AR NaOH for hydroxide ion concentrations of .01, .03, .05, .10, and .15 *M*, respectively. The .01 *M* solution was changed after every eight test discs, to insure an adequate reserve of NaOH. The water used possessed 120 p.p.m. hardness, as $CaCO_3$. Radioactivities of the soil films were determined with a thin-window Geiger tube in a Tracerlab SC 9 E shielded manual sample changer, coupled to an SC 1000 scaler.

## RESULTS AND DISCUSSION

Hydroxide ion is undoubtedly involved in several reactions with these heterogeneous milk films but, kinetically, the over-all effect of hydroxide ion on cleaning should be closely related to a bimolecular reaction that can, after collision, proceed in different directions.

In the first analysis, hydroxide ion might be expected to limit its action to the exposed surface of the soil and, in effect, remove one layer of soil after another, exposing a fresh layer each time. If this were true, the rate of soil removal would be independent of the amount of soil until less than a monomolecular layer remained. Figure 1 indicates this is not the case, and that at a constant hydroxide ion concentration, the rate of soil removal decreases as the soil is removed. This could be interpreted as representing a saturation type phenomenon and, at least in the first stages, the reaction is probably first order in respect to soil. To avoid complications arising from a change in reaction rate with disappearance of the soil, determinations were, for the most part, limited to the disappearance of ca. 60% of the soil film.

It seems reasonable that under these test conditions, where the amount of soil is quite small in respect to the over-all amount of hydroxide ion available, and the latter is large enough that its concentration can be considered constant, the rate of soil removal also is probably first order in respect to hydroxide ion. Under these circumstances, the rate of soil removal could be expressed as

$$-\frac{dS}{dt} = k\,(S)\,(OH^-)$$

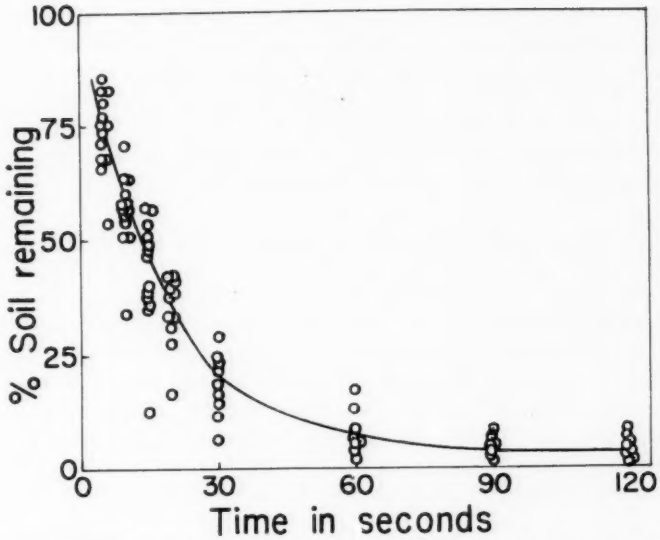


FIG. 1. Disappearance of soil with respect to time. $(OH^-)$, 0.03 $M$; temperature, 46° C.; Re, 550,000.

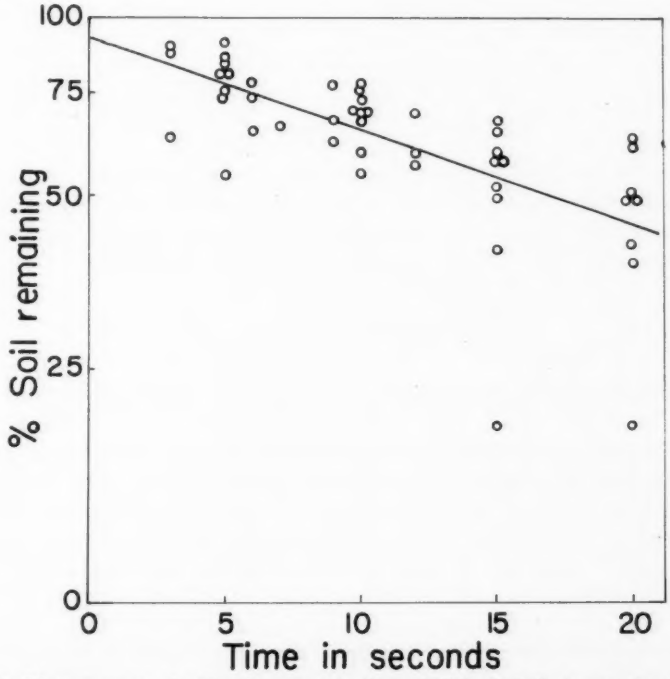


FIG. 2. Disappearance of soil with respect to time. $(OH^-)$, 0.01 $M$; temperature, 46° C.; Re, 550,000.

where $S$ denotes soil, expressed as per cent of the original deposit. There are theoretical objections to this approach, inasmuch as the unreacted soil is not in solution, but the results obtained and their high coefficients of correlation are ample justification. Ideally, the validity of this expression as regards hydroxide ion could be tested by constructing linear plots of $S$ against $t$ for various hydroxide ion concentrations. The tangents of these curves at some one value of $S$ would equal $k\ (OH^-)$; this method would eliminate the effect of (S) on the expression.

However, determining the position of these curves is difficult because the data do not yield single precise points for a given value of $t$, but possess considerable scatter. By assuming first order kinetics and resorting to semilog plots, the best-fit lines can be calculated for the data. This approach was used in constructing Figures 2–6. The lines do not extrapolate through 100% soil because of a small inherent error in timing. The abscissae, time in seconds, represent pumping time under conditions of high turbulence. When the pump is shut off at the conclusion of a run, the fluid in the line drains under flow conditions that are essentially laminar. The correlation coefficients for the best-fit lines are listed in Table 1; each is highly significant. Included are the slopes of these best-fit

TABLE 1

Correlation coefficients and slopes for Figures 2–6

| Figure | Hydroxide ion concentration | Slope | Correlation coefficient |
|---|---|---|---|
| 2 | .01 *M* | .016 | .65 |
| 3 | .03 *M* | .022 | .89 |
| 4 | .05 *M* | .027 | .98 |
| 5 | .10 *M* | .032 | .87 |
| 6 | .15 *M* | .043 | .91 |

lines, as $-d \log S/dt$ at specific hydroxide ion concentrations. In Figure 7, the slopes are plotted as a function of hydroxide ion concentration. The line that fits these data best (method of least-squares) represents the activity of hydroxide ion in removing this type of milk film under these conditions.

The equation for this line can be expressed as:

$$-\frac{d \log S}{dt} = k\ (OH^-) + C$$

where the constants have the values $k = 0.179$ sec.$^{-1}$ and $C = 0.016$ moles/liter/second.

The $y$ intercept shows that soil removal is not entirely a function of hydroxide ion concentration. This is to be expected, for although we have an analogy to a bimolecular reaction, one species, $S$, will continue to disappear even in the absence of $OH^-$, due to the mechanical effects of solution turbulence, solution of soil constituents by the water phase, and other physical phenomena. The constant $k$ might be termed the molar velocity constant of hydroxide ion on this type of soil.

*Effect of temperature.* Many attempts have been made to evaluate the effect of temperature on cleaning. Results in the literature (1, 2, 3, 5, 6, 7) are not

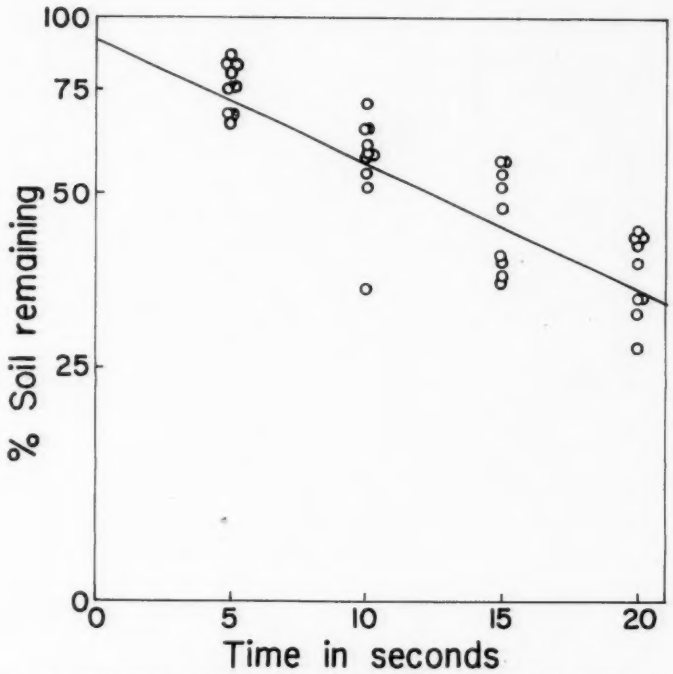


FIG. 3. Disappearance of soil with respect to time. $(OH^-)$, 0.03 $M$; temperature, 46° C.; Re, 550,000.

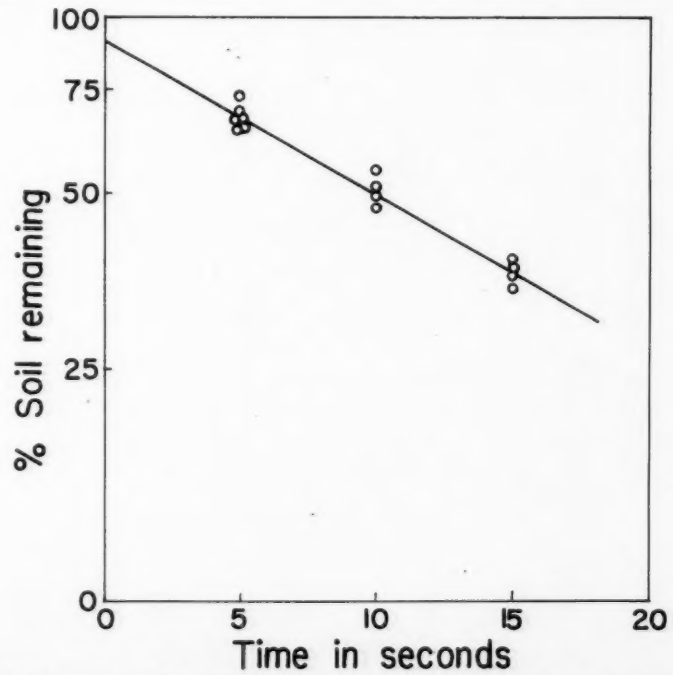


FIG. 4. Disappearance of soil with respect to time. $(OH^-)$, 0.05 $M$; temperature, 46° C.; Re, 550,000.

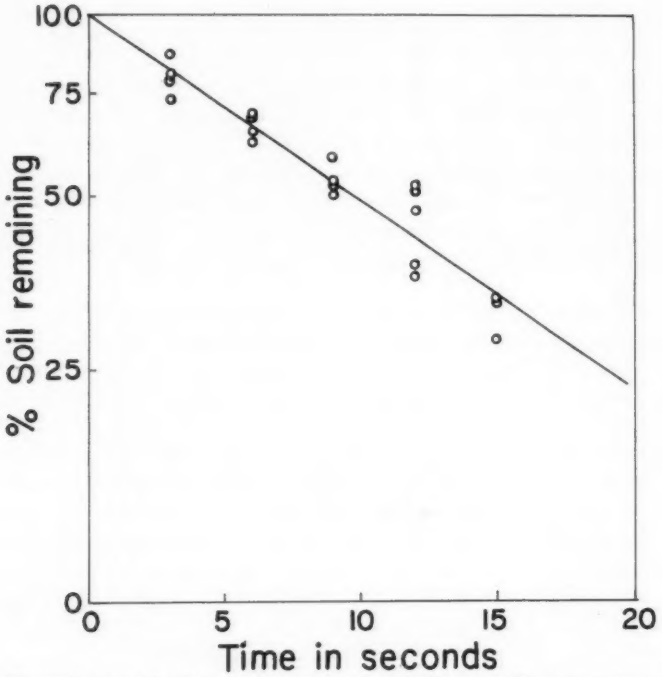


FIG. 5. Disappearance of soil with respect to time. $(OH^-)$, 0.10 $M$; temperature 46° C.; Re, 550,000.

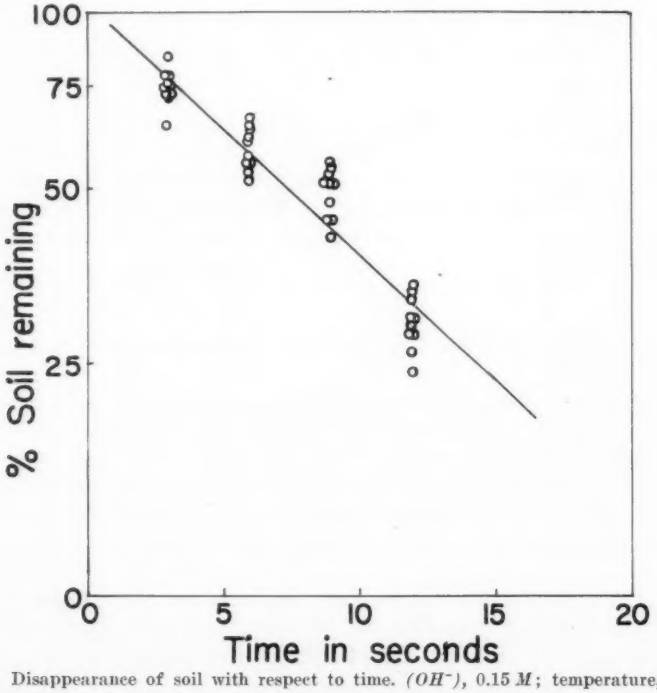


FIG. 6. Disappearance of soil with respect to time. $(OH^-)$, 0.15 $M$; temperature, 46° C.; Re, 550,000.

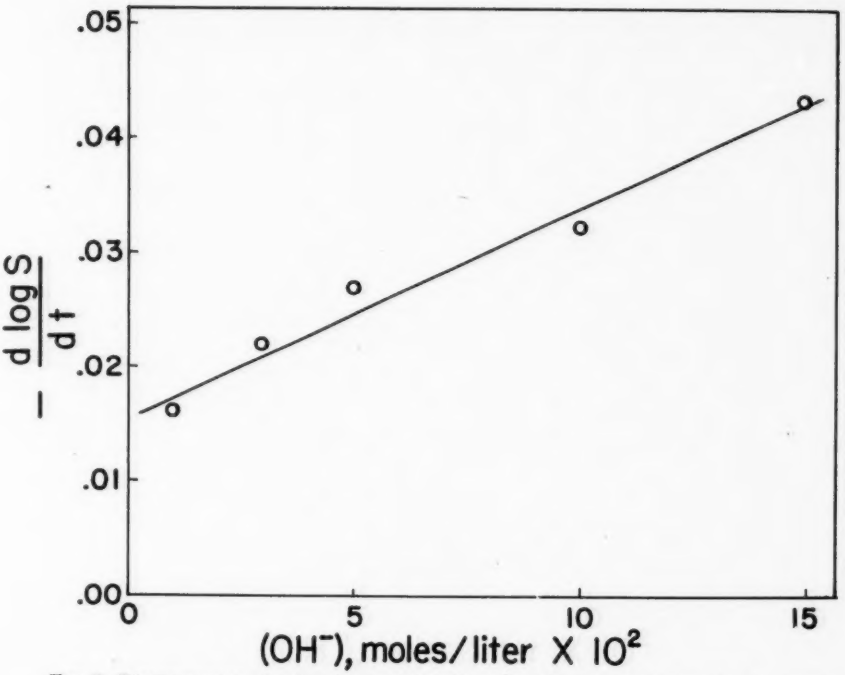


FIG. 7. Disappearance of soil with respect to time as a function of hydroxide ion concentration. Temperature, 46° C.; Re, 550,000.

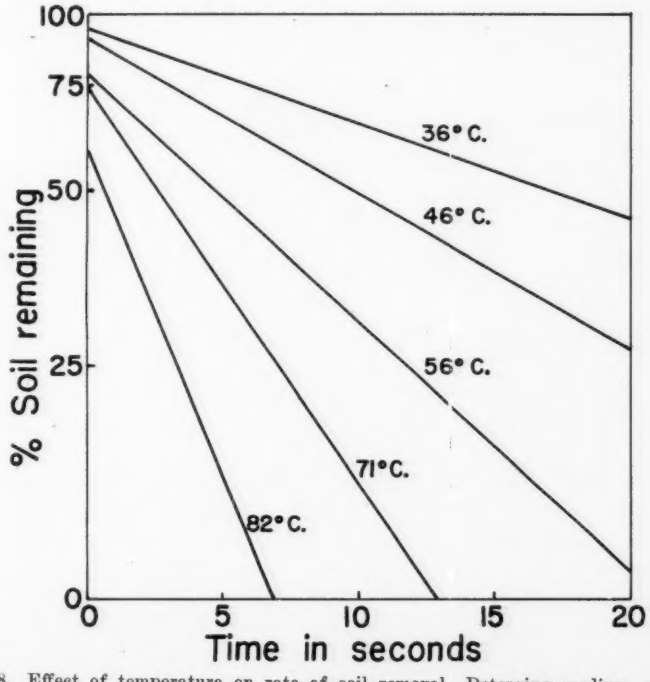


FIG. 8. Effect of temperature on rate of soil removal. Deterging medium, water; Re, 550,000.

in good agreement, largely because temperature effects could not be precisely evaluated. Series of determinations were made using .05 *M* sodium hydroxide at five different temperatures. All other experimental conditions remained the same.

Figure 8 illustrates the change in slope and $y$ intercept as the temperature of the deterging medium is increased from 36 to 82° C. (these are best-fit lines, and individual points have been omitted for clarity), and in Figure 9(a) the the apparent linearity of this increase is demonstrated by plotting log $k$ against the reciprocal of the absolute temperature. The change in slope (Figure 8) indicates that water is not inert, nor is its effect independent of temperature; at higher temperatures, it assumes a more important role.

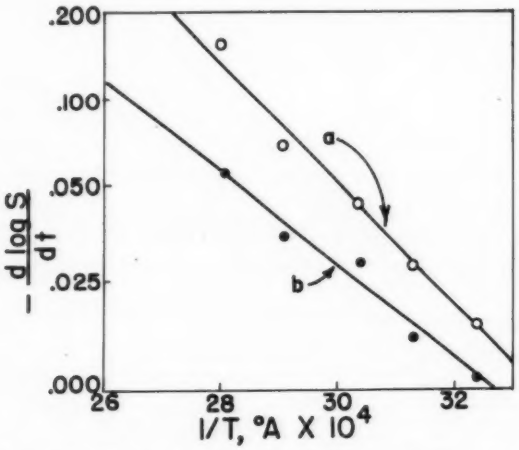


FIG. 9. Effect of temperature on rate of soil removal. Re, 550,000. a—.05 *M* ($OH^-$); b—water.

To properly evaluate the temperature variable, apparently it is necessary to set up an expression that takes into account the effect of temperature on (1) soil removed by the action of hydroxide ion, and (2) soil removed by action of the solvent, water:

$$-\frac{dS}{dt_{over\text{-}all}} = \frac{dS}{dt_{OH^-}} + \left(-\frac{dS}{dt_{HOH}}\right)$$

$-\frac{dS}{dt_{HOH}}$ can be evaluated for 46° C. by extrapolating Figure 7 to $(OH^-) = 0$, and at other temperatures by determining directly the rate of soil removal in the absence of hydroxide ion. Using techniques described above, series of determinations were made at several temperatures. Results are shown in Figure 9b.

From the formulas of the best-fit lines for these data [(a) $y = 7.898 - .0198\,x$; (b) $y = 6.557 - .0149\,x$)], it follows that

$$-\frac{d \log S}{dt_{over\text{-}all}} = 7.898 - \frac{.0198}{T_A}, \text{ and}$$

$$-\frac{d \log S}{dt_{HOH}} = 6.557 - \frac{.0149}{T_A}$$

Assuming that the cleaning effects of hydroxide ion and water are additive,

$$-\frac{d \log S}{dt_{OH^-}} = 1.341 - \frac{.005}{T_A}$$

these data point up the fact that in this range of concentration of hydroxide ion, water, far from being inert, is the most active detergent in the system (unless the comparison is on a mole-per-mole basis). While increasing temperature increases the effectiveness of both hydroxide ion and water, the increase is greater in the former. Solutions of hydroxide ion exhibit a $Q_{10}$ of 1.6 on this type of soil.

## CONCLUSIONS

It would appear that the phenomenon of cleaning can be treated from the standpoint of chemical kinetics, and that $k$ values, or molar velocity constants, can be determined for the reaction of individual detergent fractions with specific types of soil. Determination of these constants permits a more precise evaluation of the effect of temperature on cleaning, the study of compatibility, incompatibility of complementary interaction of detergent fractions and, ultimately, should allow selection of a detergent or combination of detergents to best remove specific types of soil.

## ACKNOWLEDGMENTS

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# ACTION OF PURIFIED MILK PHOSPHATASE ON PHOSPHOSERINE AND ON CASEIN

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**SUMMARY**

The action of a purified alkaline phosphatase from milk on casein and phosphoserine has been studied. The milk phosphatase preparation has no proteolytic action on casein, but it splits inorganic phosphate from both casein and phosphoserine. The action of the milk phosphatase was studied at pH 8 to 9.5 with several concentrations of phosphoserine. The optimum action was at pH 9.5 or higher. Values for the kinetic constants $K_M$ and $V_{max.}$ were calculated from the data. The action of the milk phosphatase on phosphoserine was similar to its action on other simple phosphate esters. The milk phosphatase was considerably less reactive with casein (about 200 times more phosphatase was required for comparable release of phosphate), and the greatest activity was at pH 6 to 7. Reasons are given for not necessarily postulating a second enzyme for this activity, but rather that it might result from the complex nature and size of the casein molecule. Both calcium-sensitive α-casein and whole casein were dephosphorylated at comparable rates. The experiments were carried to 80% dephosphorylation of the casein, and presumably complete dephosphorylation could be accomplished with this phosphatase preparation. It is suggested that some dephosphorylation of casein might occur in milk and that special precautions may be required to obtain casein preparations of maximum and constant phosphorus content.

The availability of a purified, highly active milk phosphatase (23, 26) suggested that its action on casein be studied, since a number of other phosphatases (9, 11, 17) have been found to dephosphorylate casein. At the same time, the action of the milk phosphatase on serine phosphate has been studied, since most or all of the phosphate contained in casein is probably esterified to the OH group of this amino acid (8, 9, 18) or to the amino acid threonine (3).

## MATERIAL AND METHODS

*Whole casein.* Whole casein was prepared by acidification of skimmilk to pH 4.5 with *N* HCl. The precipitate was washed four times with water, twice dissolved and reprecipitated with acid, then dried with absolute ethanol and ether (6).

*α-Casein, calcium-sensitive.* A calcium-sensitive α-casein was prepared from whole casein by a modification (24) of the fractionation procedure in aqueous urea solutions (7).

*Preparation of alkaline phosphatase from milk.* The alkaline phosphatase was prepared from skimmilk by the method previously described (23, 26). The preparation used in the present studies contained seven units of phosphatase activity per milligram of solids. A unit of phosphatase is defined as the amount which causes the liberation of 1 $\mu M$ phenol in 5 min. at 38° C. The unit of activity

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defined here conforms to a recommended practice (10) and is about 300 times larger than the unit previously used (23). The phosphatase preparation contains about 2,500 units per milligram of the previously defined units (26). The dried phosphatase preparation was suspended in water to a concentration of 0.5% for the casein experiments, 0.1% for the phosphoserine experiments. The suspension was centrifuged and the supernatant solution, which contained all of the phosphatase activity, had a protein content (nitrogen × 6.25) equivalent to 70% of the dry weight of the phosphatase preparation, and the specific activity is about ten units per milligram of protein in solution.

*Assay of alkaline phosphatase.* The assays were run at 38° C. in an ethanolamine-HCl buffer of pH 9.8 with phenylphosphate as the substrate (25). The liberated phenol was determined colorimetrically (25) in the Beckman Model B spectrophotometer at a wave length of 650 mμ. The phenol equivalence was read from a standard curve relating phenol and color.

*Action of the phosphatase on phosphoserine.* The action of the phosphatase was determined by measuring the inorganic phosphate released from phosphoserine (commercial source) in 30 min. at 38° C. Two milliliters of the incubation mixture contained 1.6 ml. veronal-acetate buffer (12) in the pH range 8.0 to 9.5, 6 $\mu M$ $MgCl_2$, 10 γ milk phosphatase, and 0.0125 to 5 $\mu M$ phosphoserine. The reaction was stopped by the addition of 0.5 ml. of 0.38 $N$ $H_2SO_4$. The inorganic phosphorus released was determined by the method of Dryer *et al.* (4). The optical density was measured in the Beckman DU spectrophotometer at 345 mμ and the phosphate estimated from a standard curve relating optical density and phosphate.

*The action of phosphatase on caseins.* The caseins, either whole casein or the calcium-sensitive α-casein, were dissolved using 0.1 $N$ NaOH to obtain the approximate pH desired. Veronal-acetate buffer (12) of the desired pH was added and the pH was readjusted, if necessary, with 0.1 $N$ NaOH or HCl. Five milliliters of incubation mixture contained 1.25 ml. buffer, 15 $\mu M$ $MgCl_2$, 25 mg. casein, except where stated otherwise, and usually 5 mg. of the milk phosphatase. The solutions were incubated for various times at 38° C. The reaction was stopped by adding 1 ml. incubation mixture to 1 ml. 0.02 $M$ silicotungstic acid, then 2 ml. of 20% trichloroacetic acid was added and the mixture centrifuged. The inorganic phosphate was determined on 2 ml. of the supernatant solution by the method of Sumner (21). The optical density of the developed color was measured in the Beckman DU spectrophotometer at 650 mμ.

## RESULTS

The conditions for the optimum interaction of the alkaline phosphatase of milk with the substrate phosphoserine were investigated, with the thought that the results might suggest the best conditions for the interaction of alkaline phosphatase with casein. The phosphate of casein is probably bound through the hydroxyl groups of the amino acids serine or threonine, since phosphopeptides obtained from casein have the phosphate bound to the serine (8, 9, 18) or threo-

nine (3). The action of the phosphatase on phosphoserine was studied at several pH values, to determine the optimum pH, and also with several concentrations of the substrate, phosphoserine, to determine the enzyme-substrate dissociation constant, $K_M$, as a measure of the affinity between substrate and enzyme. The latter also provided data for estimating the concentration of substrate, giving the maximum velocity, $V_{max.}$, for the reaction. At a concentration of phosphoserine suitable for assay purposes, namely $125 \times 10^{-5}$ $M$ per liter, the pH optimum had not been reached even at pH 9.6. As with other alkaline phosphatase reactions, the pH optimum is a function of substrate concentration and with $25 \times 10^{-5}$ $M$ phosphoserine per liter, the optimum is at pH 9.0.

The $K_M$ and $V_{max.}$ for the action of the milk phosphatase on phosphoserine were computed from the integrated form of the Michaelis-Menten equation developed by Walker and Schmidt (22).

$$(S_o - S_t) \cdot t = -K_M (2.3 \log S_o/S_t) \cdot t + V_{max.}$$

$S_o$ is the initial substrate concentration, and $S_t$ is the substrate concentration after the action of the enzyme for time $t$. By a plot of $(S_o - S_t) \cdot t$ versus $(2.3 \log S_o/S_t) \cdot t$, a straight line is obtained. The slope is $-K_M$ and the intercept on the $(S_o - S_t) \cdot t$ axis is $V_{max.}$ The use of this equation made it possible to include data at low substrate concentrations for the calculation of $K_M$ and $V_{max.}$, a substrate region where reaction rates are linear for only a brief time. The results are presented in Figure 1, where $pK_M$ $(= 1/\log K_M)$ is plotted against pH, and in Figure 2, where $V_{max.}$ is plotted against pH. The large $pK_M$ value at pH 8 means that the substrate concentration has to be less than $2.5 \times 10^{-5}$ $M$

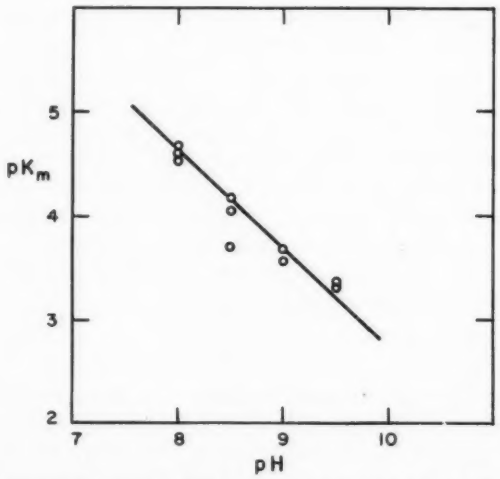


FIG. 1. The enzyme-substrate dissociation constant $(pK_M = \log 1/K_M)$ for the action of the milk alkaline phosphatase on the substrate phosphoserine at various pH values. $K_M$ is expressed in moles per liter.

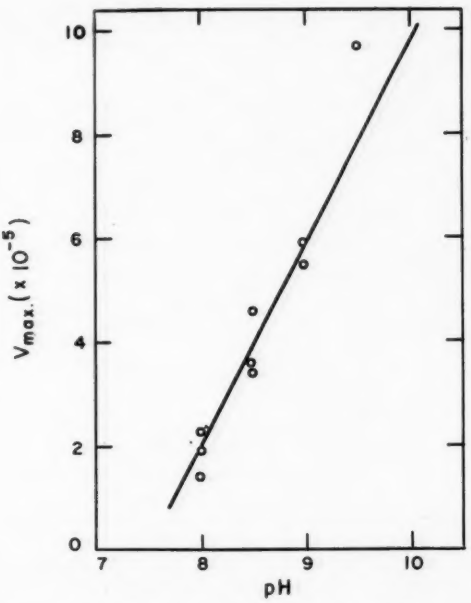


FIG. 2. The maximum velocity, $V_{max.}$, for the action of the milk phosphatase on phosphoserine at various pH values. $V_{max.}$ is expressed in moles per liter per 5 min.

per liter before the reaction becomes dependent on substrate concentration. This made it impractical to estimate the $K_M$ values at pH values below eight, since these concentrations of inorganic phosphate released were too small to be estimated accurately.

The results with phosphoserine, although of general interest, did not serve as a guide for the action of the milk phosphatase on casein. The milk phosphatase preparation did dephosphorylate casein, but much larger concentrations (about 200 times) of phosphatase were required than for phosphoserine, and dephosphorylation of casein was more rapid at pH 6 than at pH 10. A detailed comparison of the action of the milk phosphatase on the two substrates will be discussed later.

Both whole casein and calcium-sensitive $\alpha$-casein were dephosphorylated to about the same extent. The results with the two caseins and several concentrations of the milk phosphatase are shown in Figure 3. The reason for the nonlinear release of phosphorus as the amount of phosphatase is increased is not clear. It was not due to a decrease in the phosphatase activity, for all of the activity (tested with the substrate phenylphosphate) was still present at the end of the experiment. In fact, the casein stabilized the phosphatase, for phosphatase stored at this pH and temperature for the same length of time with no casein present had lost some activity. The ninhydrin reaction (13) applied to this

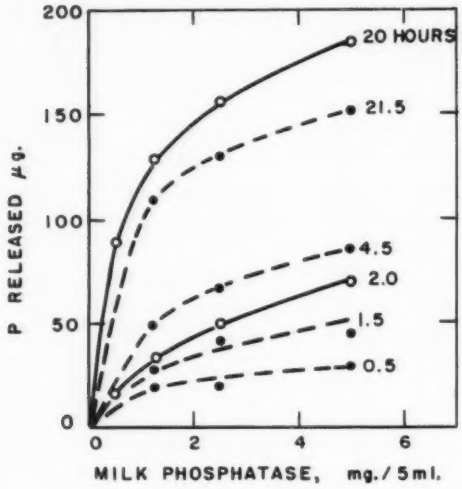


FIG. 3. Action of milk prosphatase on whole casein (pH 7.0) and calcium-sensitive α-casein (pH 6.4) for various periods of time, measured by the release of inorganic phosphate. ●——● Whole casein (177 γ casein-P in 5 ml.); ○——○ α-casein, calcium-sensitive (250 γ casein-P in 5 ml.). Numbers at ends of curves indicate hours that the phosphatase acted.

mixture remained the same after 0, 2, and 20 hr.; hence, the milk phosphatase preparation contained no proteolytic activity for the conditions used.

The data in Figure 4 show the influence of time and pH on the dephosphorylation of calcium-sensitive α-casein by the milk phosphatase. The maximum rate of hydrolysis results at pH 6 and 7. The shape of these curves—rapid initial hydrolysis followed by a considerably slower rate—is characteristic of this dephosphorylation.

The effect of the concentration of casein on the dephosphorylation activity of the milk phosphatase is shown in Figure 5 for pH 6.5 and 9.0. $K_M$ and $V_{max.}$ values were calculated by the procedure described earlier for phosphoserine. Straight lines were obtained when $(S_o - S_t) \cdot t$ was plotted against (2.3 log $S_o/S_t \cdot t$; hence, for these conditions, the catalytic activity of the phosphatase appears to represent the usual summation of zero- and first-order rates.

## DISCUSSION

Chemical properties of phosphoserine have been described (16) and a few reports of the action of phosphatases on this compound have appeared. A preparation from calf intestinal mucosa will dephosphorylate phosphoserine as rapidly as it does glycerophosphate (19), presumably due to the alkaline phosphatase which is present in intestinal mucosa in large amounts. Phosphatases also have been reported (15, 20) that are specific for phosphoserine, acting only on this substrate. They will also phosphorylate serine.

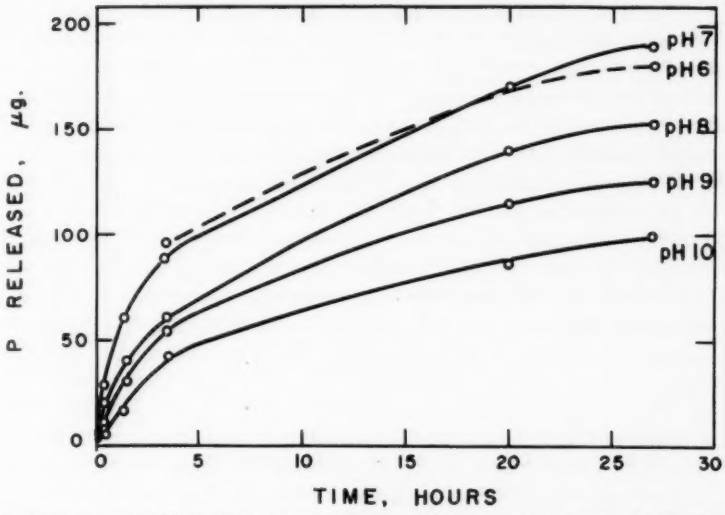


FIG. 4. Action of milk phosphatase on calcium-sensitive α-casein at several pH values, for various periods of time, measured by the release of inorganic phosphorus. The amount of milk phosphatase in this experiment was 2.5 mg. in the 5-ml. reaction mixture.

The present studies show that the action of the milk alkaline phosphatase on phosphoserine is very similar to its action on other simple phosphate esters. Phosphoserine and phenylphosphate are hydrolyzed equally rapidly by the milk phosphatase (23); with phosphoserine, however, the phosphatase has the same

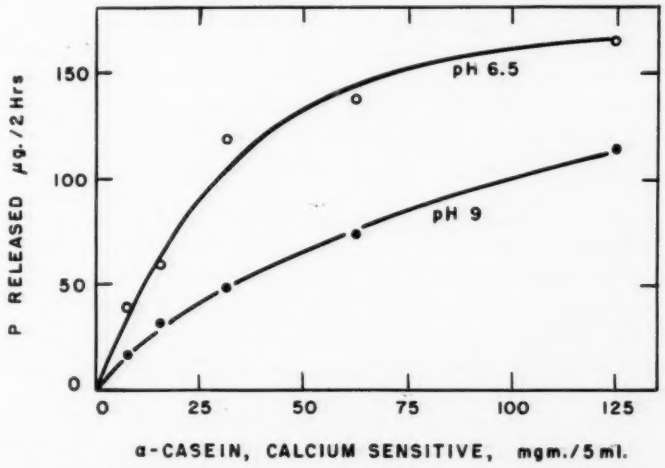


FIG. 5. Action of milk phosphatase on calcium-sensitive α-casein with several concentrations of the casein at pH 6.5 and 9.0, measured by the release of inorganic phosphorus in 2 hr.

$pK_M$-pH values as with *o*-carboxyphenylphosphate (23). The milk phosphatase, acting on phosphoserine, also has its greatest activity at pH 9.5 or above. The milk phosphatase preparation used in the present study contained no acid phosphatase, that is, a phosphatase active at pH 4, which has been reported in milk (14). Large amounts of the milk phosphatase were incubated at 35° C. at pH 4.0 with phenylphosphate and with phosphoserine with negative results.

The information about the action of the milk phosphatase on phosphoserine provided no guide to its action on casein. The milk phosphatase does dephosphorylate casein, but large amounts of the phosphatase are required, and the most marked difference is that dephosphorylation is more rapid at pH 6 to 7 than at pH 9.5. As noted above, the phosphatase preparations did not contain an acid phosphatase, but the above pH data suggest that a second dephosphorylating enzyme might be present. Previous studies (26) had showed that the purified milk phosphatase preparations contained an enzyme-hydrolyzing nucleic acid, and there well might be other phosphorylitic enzymes in the milk phosphatase preparations also. The pH data, however, do not necessarily require that the enzyme-dephosphorylating casein be different from the enzyme-hydrolyzing phosphoserine and other simple phosphate substrates. The casein is a large molecule and there might be a local charge on its molecule at the higher pH values that would hamper enzyme-substrate union and consequent dephosphorylation of the casein.

At pH 6, on the other hand, the unfavorable charge might be absent, thus permitting action of the phosphatase, although at a low level. A phosphatase preparation from calf intestinal mucosa will dephosphorylate casein (11) but here, too, although it is a potent source of alkaline phosphatase, the greatest activity on casein is at pH 7.4. A kinetic analysis of the action of the milk phosphatase on casein (Figure 5) shows that this action has a greater affinity between enzyme and substrate (smaller $K_M$ value) at pH 6.5 than at pH 9.0. In this respect, the effect of pH is similar to the effect on the action of the phosphatase on phosphoserine. The actual values for $K_M$ are 1.5 m*M* casein-P per liter (0.5% casein) at pH 6.5, and 4.5 (1.5%) at pH 9.0. These values are of the same magnitude as others have reported for enzymatic dephosphorylation of casein. The value for the spleen phosphatase acting on casein at pH 5.5 was 3 m*M* casein-P per liter (9).

Calcium-sensitive $\alpha$-casein was chosen for the present studies to have a casein that was free of other interacting components. This choice was motivated by the report (17) that whole casein was very poorly dephosphorylated by phosphatase, whereas the separated $\alpha$- and $\beta$-caseins were readily dephosphorylated. Recent studies (9, 11), however, have not confirmed this claim, and the present studies show that the calcium-sensitive $\alpha$-casein and whole casein were dephosphorylated at comparable rates.

The results of the present study raise the question whether the action of the phosphatase in milk might lead to caseins of variable phosphate content, depending on the time of isolation after milking and other factors. The amount of phosphatase used in the present studies, although large, to obtain a readily measur-

able dephosphorylation, is only about ten times greater than the concentration found in milk. There is about 0.7 unit (defined above) of phosphatase per milliliter of milk (0.7 unit to about 30 mg. casein); whereas, the purified phosphatase contained seven units per milligram and, as shown in Figure 3, 1 mg. of this phosphatase gives a marked dephosphorylation of 25 mg. of casein.

It is well known that the inorganic phosphate in milk increases on standing, but analysis indicates that all of this increase seems to be accounted for by a decrease in the soluble, low molecular weight phosphate esters in milk (5). The relatively large contribution of this fraction to an increase in the inorganic phosphate might, however, mask a contribution from dephosphorylation of casein. Changes in the phosphatase content of cow's milk by the administration of thyroxine or thiouracil bring about changes in the phosphorus content of the casein (1). Thyroxine decreases the phosphatase in milk and the phosphate content of the casein goes up; thiouracil increases the phosphatase content of milk (as much as threefold above normal) and the phosphate content of casein goes down. A somewhat similar pattern emerged from changes in the phosphatase during the course of lactation (2). The authors (1) attributed the changes in the phosphate content of casein to metabolic changes within the mammary gland. The present studies, however, suggest that the change in the phosphate content of casein may have taken place after the milk was formed. Also, it appears that special precautions will be required to obtain casein preparations of maximum and constant phosphate content.

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# PSYCHROPHILES IN MILK HELD TWO DAYS IN FARM BULK COOLING TANKS[1]

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## SUMMARY

Predominant psychrophilic bacteria were isolated from 174 samples of every-other-day milk from bulk milk cooling tanks from 12 farms producing Grade A milk. The 220 isolates so obtained were identified as to genus and species as far as possible. For the most part they fell into the genera *Aerobacter, Alcaligenes, Arthrobacter, Flavobacterium, Micrococcus,* and *Pseudomonas.* Species of these genera are listed. Not all of the species could grow in pasteurized milk within three days at the cooling tank temperature of 38° F. (3.3° C.), although they formed colonies in seven days at 5° C. on plate count agar, and most of them could grow in pasteurized milk at 45° F. (7.2° C.) All of the species grew little, if any, in raw milk in the cooling tanks at 38° F.

During barn feeding and holding of the cows, *Arthrobacter* species usually were predominant in the every-other-day milks, with *Pseudomonas* second in frequency and *Micrococcus* third. While the cows were on pasture, bacteria of the genus *Flavobacterium* were most often predominant, followed by *Arthrobacter,* and *Alcaligenes* first was detected. There was an average sevenfold increase in total numbers of psychrophiles when the cows went on to pasture from barn housing. There seemed to be a tendency for a certain genus of psychrophile to predominate in milk from a given farm, although the method of holding and feeding, barn versus pasture, had an important influence.

Marth and Frazier (2) reported that the predominant psychrophiles in most high-count, raw market milk handled chiefly in pipeline milkers were in the genera *Pseudomonas, Alcaligenes, Achromobacter,* and *Flavobacterium.* By contrast, the milks in the present studies were low-count, for the most part, and did not contact farm pipelines. The genus *Micrococcus* was not reported by Marth and Frazier, but often is listed among the genera containing psychrophiles.

## METHODS

Incidental to the estimation of psychrophilic plate counts on every-other-day milk from the bulk cooling tanks of 12 farms from the Chicago milkshed (1), plates became available for the isolation of psychrophilic bacteria. Psychrophiles in this instance were considered to be bacteria that formed visible colonies in plate count agar (*Standard Methods for the Examination of Dairy Products,* 10th ed. 1953) after incubation for seven days at 5° C. The milk from the 12 farms was produced to meet Grade A standards and, for the most part, had low standard plate counts and psychrophilic plate counts. Arithmetic means of the standard plate counts of all every-other-day samples of patrons' milks ranged from 4,700 to 110,000 per milliliter and had an over-all average of about 27,000 per milliliter. Arithmetic means of the psychrophilic plate counts ranged from 350 to 4,800 per milliliter and averaged 2,800 per milliliter.

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[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] At present with The Quaker Oats Co., Barrington, Ill.

All farm bulk cooling tanks were refrigerated, so as to maintain a milk-storage temperature of approximately 38° F. (3.3° C.), except briefly at milking time. Two-day-old milk samples were collected at the 12 farms and iced during transportation to the laboratory, where they were plated for the psychrophiles. From each sample of milk a single plate culture of the highest dilution showing appreciable growth was chosen and examined for predominant colonies. One colony of each predominant kind was picked and purified, and an attempt was made to identify the bacterium. Altogether, 220 isolates were obtained from 174 samples of every-other-day milk from the 12 farms. The bacteria were classified into genera and, if possible, into species, from descriptions outlined in Bergey's *Manual of Determinative Bacteriology*, 7th ed. 1957. Nine isolates were not identified as to genus.

## RESULTS

It will be observed in Table 1 that *Arthrobacter* was predominant most often, making up about 37% of the isolates, and *Flavobacterium* next most often, comprising about 29% of the isolates. Next came about 17% of *Pseudomonas*, followed by still fewer bacteria of the genera *Micrococcus* and *Aerobacter*. Only twice was *Alcaligenes* predominant. The species tested under the various genera fitted the descriptions in Bergey's *Manual*, except for an occasional minor characteristic.

A test was made of the ability of representatives of some of the above genera to grow within three days at 38° F. (3.3° C.) in pasteurized milk. None of the species of *Arthrobacter*, *Micrococcus*, or *Flavobacterium* tested was able to grow. Fairly rapid growth of *Alcaligenes marshallii*, *Pseudomonas segnis*, *Pseudomonas convexa*, and *Pseudomonas cohaerens* was observed, and almost as good growth of *Aerobacter aerogenes*. *Pseudomonas schuylkilliensis* showed a two-day lag before

TABLE 1

Frequency of isolation of genera and species of psychrophiles from every-other-day milk

| Genus | Species | Total No. of isolates | Percentage of total isolates |
|---|---|---|---|
| *Arthrobacter* | | 81 | 36.8 |
| | *A. simplex* | 59 | 26.8 |
| | *A. citreus* | 11 | 5.0 |
| | Unidentified | 11 | 5.0 |
| *Flavobacterium* | | 63 | 28.6 |
| | *F. aquatile* | 61 | 27.7 |
| | *F. suaveolens* | 2 | 0.9 |
| *Pseudomonas* | | 37 | 16.8 |
| | *P. schuylkilliensis* | 8 | 3.6 |
| | *P. convexa* | 7 | 3.2 |
| | *P. cohaerens* | 17 | 7.7 |
| | *P. putrefaciens* | 2 | 0.9 |
| | *P. segnis* | 3 | 1.4 |
| *Micrococcus* | | 16 | 7.4 |
| | *M. candidus* | 9 | 4.1 |
| | *M. caseolyticus* | 3 | 1.4 |
| | *M. conglomeratus* | 4 | 1.8 |
| *Aerobacter* | | 13 | 5.9 |
| | *A. aerogenes* | 9 | 4.1 |
| | *A. cloacae* | 4 | 1.8 |
| *Alcaligenes* | *A. marshallii* | 2 | 0.9 |
| Unidentified | | 9 | 4.1 |

initiating growth, and *Pseudomonas aquatile* failed to grow. Most of the species unable to grow within three days at 38° F. (3.3 C.) could grow in pasteurized milk at 45° F. (7.2° C.). It should be reported, however, that even those species able to grow well at 38° F. in pasteurized milk apparently grow little, if at all, in the raw milk in the cooling tanks, for psychrophilic plate counts averaged as low for one-day milks as for every-other-day milks.

It was observed that a certain genus of psychrophile tended to predominate in milk from a given farm, although, as will be shown, the method of feeding, barn versus pasture, had an important influence. Milk from seven farms had an over-all predominance of bacteria of *Arthrobacter* species, although they were predominant less often during pasture feeding than during barn feeding. Milk from three farms had *Flavobacterium* species predominant most often; one farm had mostly *Pseudomonas* and one chiefly *Aerobacter*.

During the period of the experiments (March 3 to June 8), the cows were changed from barn feeding to pasture feeding. Table 2 shows the predominant genera found in the every-other-day milk samples during the two periods. During the time that the cows were fed in barns, more isolations were made of bacteria of the genus *Arthrobacter* than of any other genus, with *Pseudomonas* second in frequency and *Micrococcus* third. On the other hand, after the cows went on to pasture, bacteria of the genus *Flavobacterium* were predominant, followed by *Arthrobacter;* and *Alcaligenes* first was detected in two samples. Total average numbers of psychrophiles increased from 920 per milliliter during barn feeding to 6,700 per milliliter during pasture feeding.

TABLE 2

Comparison of predominant genera of psychrophiles in every-other-day milk from 12 patrons during barn feeding and pasture feeding

| Psychrophiles | Percentage of total cultures isolated | |
|---|---|---|
| | Barn feeding[a] | Pasture feeding[b] |
| *Arthrobacter* spp. | 51 | 24 |
| *Flavobacterium* spp. | 6 | 56 |
| *Micrococcus* spp. | 10 | 5 |
| *Pseudomonas* spp. | 25 | 9 |
| *Aerobacter* spp. | 8 | 4 |
| *Alcaligenes* spp. | 0 | 2 |

[a] Eight-week period.
[b] Following 4-wk. period.

## DISCUSSION

Apparently there was little, if any, growth of psychrophiles during holding in the cooling tank in most of the milk samples tested. Therefore, the higher numbers present in the milk during pasture feeding must have been caused primarily by contamination additional to that received in the barn at milking while the cows were being held in the barn. Present results indicate that this additional contamination consisted chiefly of yellow bacteria that have been classified here as *Flavobacterium* species. According to Bergey's *Manual,* these bacteria come mainly from water or soil, and *Arthrobacter* species from soil. It

has been known for some time, however, that the surface flora of green plants contains a considerable proportion of yellow-pigmented, gram-negative, nonsporeforming rods that Wöller (3) and others have called *Bacterium herbicola* and the sixth edition of Bergey's *Manual* calls *Pseudomonas trifolii*. However, the yellow, gram-negative bacteria encountered in the present work and classified as *Flavobacterium*, were nonmotile, according to tests; whereas, all *Pseudomonas* species tested had polar flagella. It may be that *B. herbicola* is really a *Flavobacterium*, and that green plants are one source of these bacteria.

*Micrococcus* and *Arthrobacter* were found in the present study and not in that of Marth and Frazier (2). For the most part, the predominating bacteria of the every-other-day milk in the present experiments were those which predominated in the original contamination of the milk, for little, if any, growth took place. Bacteria of the two above-mentioned genera were unable to grow in the milk held at 38° F., but grew on the plates of the psychrophilic plate count. On the other hand, Marth and Frazier worked with milk most samples of which were heavily contaminated at the start, with the farm pipelines as an important source of bacteria. Also, there was growth of psychrophiles in a number of samples.

ACKNOWLEDGMENTS

Thanks are due J. C. Werren, whose plates were used for isolation of psychrophiles, and to the Associated Milk Dealers, Inc., of Chicago, Illinois, for partial support of the project.

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# RELATION BETWEEN LACTIC ACID AND DIRECT MICROSCOPIC COUNTS FOR BACTERIA IN NONFAT DRY MILK[1]

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## SUMMARY

About 50 dry milk samples with known direct microscopic counts for bacteria were analyzed for lactic acid by Ion exchange, Golden State, Davidson, and Ling methods. Precision was poor on all but the Ion exchange method. All methods gave correlation coefficients of about .8 between apparent lactic acid and bacteria counts. The Ion exchange method was found to be the most accurate and also the slowest. The Ling method was definitely the fastest. The value of lactic acid content as an estimate of bacteria count was calculated. The expected range of bacteria counts for a given amount of lactic acid was too large for lactic acid to be a satisfactory indicator of bacterial contamination. Roller-dried samples gave unexpectedly high analyses for lactic acid.

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The most obvious chemical change brought about by bacteria in milk is usually the production of lactic acid. There has been a persistent interest over the years in the relationship between bacteria counts and lactic acid production.

Hammer and Hix (4) found only a very general relationship between the rise in acidity and the numbers of organisms present in sterile milk inoculated with lactic acid bacteria. Similar results were found by Gould and Jensen (3) in random samples of milk which were taken as received at a creamery and allowed to stand at 22–24° C. until noticeable off-flavors developed.

In the present study, we have used four recently developed methods for lactic acid determination to analyze samples of nonfat dry milk of known direct microscopic counts for apparent lactic acid. About 50 spray-dried samples were analyzed in duplicate by the Golden State (7), Davidson (2), and Ling (5) procedures. Also, single analyses were run on 25 samples by the Ion exchange method (9). The reproducibility of each of the four methods was determined by statistical analyses of the data and an estimate was made of their accuracy. Correlation coefficients between the direct microscopic clump counts and the apparent lactic acid present also were calculated for each method. The approximate time required to run a set of samples of each method was recorded and the probable usefulness of lactic acid content in estimating the direct microscopic count was evaluated statistically.

## MATERIALS AND METHODS

In Davidson's method (2), the reconstituted milk is deproteinized and desugared by treatment with copper sulfate and lime. The filtrate is treated with sulfuric acid to convert lactic acid to acetaldehyde. Color is developed with copper sulfate and *p*-phenylphenol and measured in a spectrophotometer.

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[1] Presented in part before the American Dry Milk Institute Research Conference in Madison, Wisconsin, on October 13, 1958.

In the Golden State method (7), the sample is first deproteinized with tungstic acid and then carried through the Davidson procedure.

The Ion exchange method (9) involves first extracting the lactic acid from the dry milk powder with 80% methanol. The extract is then passed through the cation and anion exchange columns. The eluate from the anion column is treated with sulfuric acid and *p*-phenylphenol to develop the color which is measured in a spectrophotometer.

The above procedures were carried out essentially as described in the original references, which may be referred to for details of the procedures.

A modified Ling method (5), based on a procedure developed by the American Dry Milk Institute (1), was used in our study. A more satisfactory modification of the Ling method since has been developed by the A.D.M.I. (1).

In the procedure used, 5.38 g. of dry milk was reconstituted in 50 ml. of water and a 25-ml. aliquot taken. This was deproteinized by adding successively 10 ml. of barium chloride solution (98.8 g/liter), 5 ml. zinc sulfate solution (225 g/liter), and 10 ml. 0.66 *N* sodium hydroxide, and filtering. One milliliter of 1% ferric chloride in 0.025 *N* hydrochloric acid was added to 10 ml. of the filtrate. The color was read after 30 min. with optical densities taken at 425 and 650 mμ. The difference was used to determine the lactic acid present. The reading at 650 mμ was to correct for turbidity which frequently developed in the final solution.

The calibration curve was prepared by adding known amounts of calcium lactate to a milk sample and measuring the increase in optical density.

A filtrate prepared by the Davidson procedure was chromatographed to determine whether color-producing substances other than lactic acid were present. For this experiment, 1 g. of sample was deproteinized by the Davidson method and the solution volume made up to 100 ml. The solution was filtered and the pH of the filtrate (66 ml.) was adjusted to 4.5 and passed through the cation column, and the column washed with water. The pH of the cation effluent and combined wash water (approximately 200 ml.) was adjusted to pH 8 and passed through the anion column and the column washed. The volume of anion effluent and combined wash water was adjusted to 300 ml. and labeled nonionic fraction. The cation column was then eluted with 200 ml. *N* HCl and the anion column eluted with 200 ml. *N* $Na_2CO_3$. The color determination in the various fractions was then made as described in the Ion exchange method.

All colorimetric readings were made with a photoelectric colorimeter with matched 6-in. test tubes.

## RESULTS AND DISCUSSION

The statistical data for the various methods are summarized in Table 1. Of the four methods used, the Ion exchange method (9) is the only one capable of good reproducibility. The Ion exchange method gives distinctly lower results than the three other methods. It effectively separates lactic acid from interfering materials and is probably the only method that measures true lactic acid accurately. However, it is too slow for routine work.

TABLE 1
Statistical data for lactic acid analyses by four methods

| | Ion exchange | Ling | Davidson | Golden State |
|---|---|---|---|---|
| Number of samples analyzed | 25 | 57 | 48 | 50 |
| Coefficient of variation | 3.86% | 11.7% | 18.7% | 15.5% |
| Standard deviation between duplicates | ..... | 122 | 87 | 68.4 |
| Coefficient of correlation DMC–lactic acid | 0.828 | 0.838 | 0.810 | 0.844 |
| Regression line | $y = .895x + 181$ | $y = 1.337x + 774$ | $y = 1.229x + 233$ | $y = 1.091x + 230$ |
| Time required | 8 hr. for 4 samples | 4 hr. for 10 samples | 4 hr. for 8 samples | 6.5 hr. for 8 samples |

[a] $y$ = Lactic Acid ($\gamma$/g); $x$ = DMC (millions/g).

The Davidson method gives somewhat higher results than the Ion exchange method. The results of Ion exchange chromatography of a Davidson filtrate are shown in Table 2. These results show that the Davidson method is measuring other substances in addition to lactic acid.

TABLE 2
Fractionation of a Davidson filtrate by Ion exchange chromatography

| Fraction | Apparent lactic acid concentration |
|---|---|
| | *(mg/100-g. sample)* |
| Davidson filtrate | 118 |
| Cation fraction | 4 |
| Anion fraction | 88[a] |
| Nonionic | 31 |

[a] True lactic acid present in this fraction only.

The Golden State method (7) gave somewhat lower results than the Davidson procedure, indicating that it removed interfering materials more efficiently. The extra step made it a longer procedure than the Davidson method.

The Ling method (5), as run here, gave much higher values of apparent lactic acid than any of the three others. This is chiefly because we made no attempt to correct for materials other than lactic acid, which gave a color with ferric chloride in Ling filtrates. Other variations of the Ling procedure (1, 5) call for preparing a calibration curve based on lactic acid originally in the sample (as determined by the Davidson method) plus added lactate. This gives a calibration curve which supposedly corrects for color-producing substances other than lactic acid in the sample. The latter procedure assumes that the amount of interfering material is constant in all milk samples. This assumption is not supported by adequate evidence. The use of the Davidson procedure to determine lactic acid already in the sample also is questionable, since we have shown it to be inaccurate. Therefore, we rejected this procedure and, instead, measured the increase in optical density resulting from added lactate for preparation of the

calibration curve. It may be noted that the method of preparing the calibration curve does not affect the relationship between bacteria counts and apparent lactic acid, although it changes the absolute values of the lactic acid determination.

Even though the Ion exchange method is the only one of the four that approaches a true measure of lactic acid present in the milk samples, we felt that it would be useful to check the correlation of the results obtained by each of the four methods with direct microscopic counts for bacteria. It seemed quite possible that whatever was being measured by the methods which were not specific for lactic acid might be useful in estimating the bacteriological quality of milk. As it turned out, the correlation coefficients between apparent lactic acid and direct microscopic clump counts of bacteria were very nearly the same for all four methods. Therefore, if one is interested in estimating bacterial contamination rather than accurately measuring lactic acid, it is certainly justifiable to use the shortest available method, that described by Ling (5).

Figures 1–4 illustrate graphically the relationship between apparent lactic acid and the direct microscopic counts for bacteria. Lactic acid is plotted against the direct microscopic counts for each of the four methods, and the best possible straight line for the data as calculated by the method of least-squares is shown for each plot. A general and approximately linear increase in lactic acid is observed as the bacteria count increases.

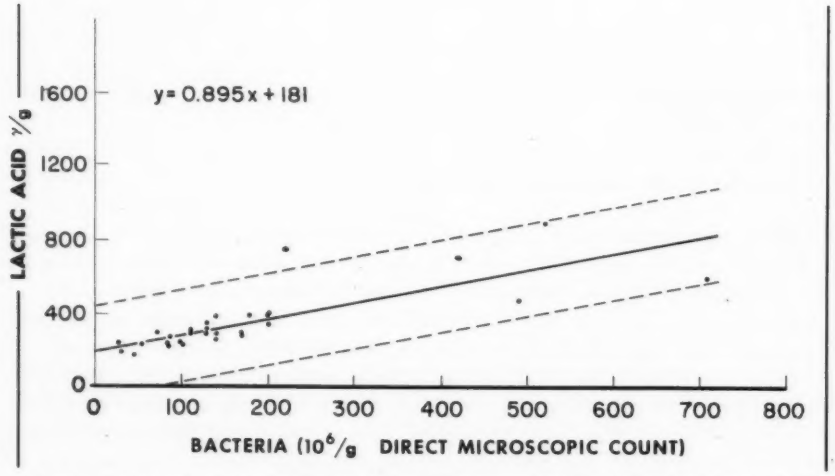


FIG. 1. Lactic acid as determined by the Ion exchange method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

The 95% confidence interval also was calculated (8) and is shown on the graph. This gives the ranges within which 95% of the experimental points may be expected to fall and was calculated to give an idea of the accuracy with which bacteria counts can be estimated from the amount of lactic acid present. For a

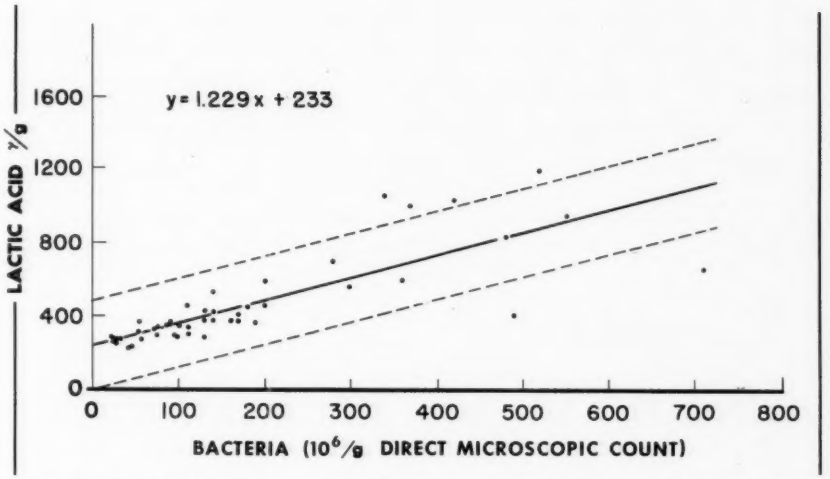


FIG. 2. Lactic acid as determined by the Davidson method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

given amount of lactic acid, the average value of the direct microscopic count may be found on the regression line and the expected range of values is shown by the 95% confidence interval lines. For the Ling method, the confidence interval is ± 192 million; for the Davidson ± 235 million; for the Ion exchange ±245 million; and, for the Golden State method ± 210 million. This very wide range

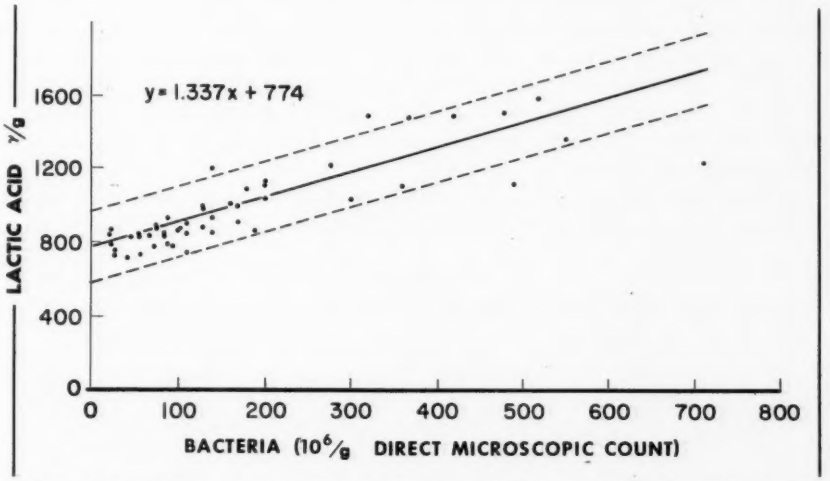


FIG. 3. Lactic acid as determined by the Ling method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

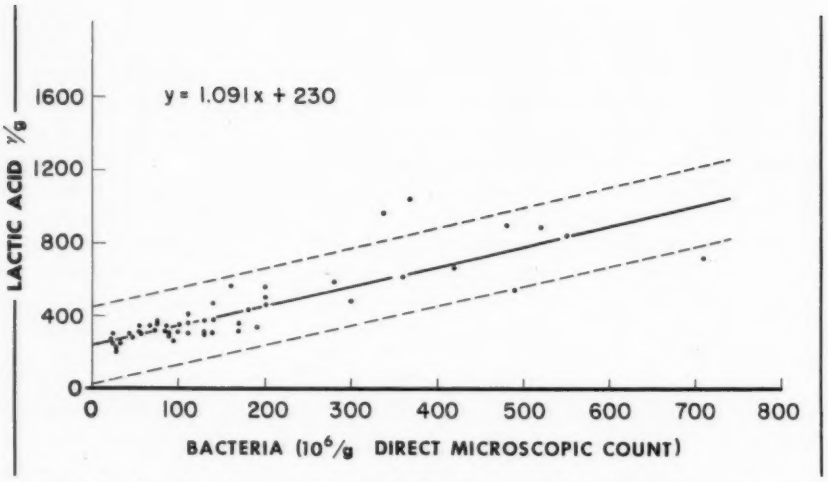


FIG. 4. Lactic acid as determined by the Golden State method vs. direct microscopic counts. Solid line—calculated regression line. Dashed lines—95% confidence limits.

of direct microscopic counts expected for a given level of lactic acid means that lactic acid content is of limited practical value for estimating numbers of bacteria present.

It is tempting to theorize that much of the observed scatter is due to the inaccuracy of the direct microscopic counts for bacteria. However, the results reported by earlier workers show similar variability (3, 4). Considering the variety of pathways to carbohydrate metabolism of bacteria (10), commonly found in milk, it seems unreasonable to expect better correlation between bacteria counts and lactic acid than is reported here. An additional variable is the formation of various acids including lactic when skimmilk is heated as shown by Morr *et al.* (6). Presumably, the extent of heat treatment would affect the amount of lactic acid from this source, further obscuring any relationship between lactic acid and bacteria counts.

Roller-dried samples gave much higher analyses for lactic acid than reasonably could be expected on the basis of their bacteria counts. The results were,

TABLE 3

Comparison of lactic acid found in spray- and roller-dried samples by the Golden State method

| Roller-dried | | | Spray-dried | | |
|---|---|---|---|---|---|
| No. | Direct microscopic count | γ/g Lactic Acid | No. | Direct microscopic count | γ/g Lactic Acid |
| | *(millions/g)* | | | *(millions/g)* | |
| 1 | 13 | 1,300 | 1 | 24 | 250 |
| 2 | 34 | 970 | 2 | 31 | 263 |
| 3 | 44 | 1,075 | 3 | 44 | 220 |
| 4 | 56 | 1,050 | 4 | 56 | 263 |

therefore, not included in the above data. Apparent lactic acid content of roller-dried samples and that of spray-dried samples of comparable bacteria count are compared in Table 3. Milk is subjected to much more severe heating in the roller-drying process than in spray drying. The lactic acid in this case probably comes from thermal decomposition of the milk, as previously discussed.

## ACKNOWLEDGMENTS

The authors wish to express their appreciation to James Dizikes and others of the Chicago USDA Agricultural Marketing Service dairy laboratory for their cooperation in providing the dry milk samples of known bacterial count used in this study.

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# RELATIONSHIPS OF HEAT TREATMENT, SOLIDS-NOT-FAT, AND CALCIUM CHLORIDE TO THE CURD TENSION OF SKIMMILK[1]

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## SUMMARY

The effect of high-heat treatments in reducing the curd tension of milk has been further demonstrated. It has also been shown that the curd tension of milk heated as high as 165° F. for 30 min. could be restored, i.e., raised to approximately 54 g. (an average value for milk containing 9% solids-not-fat and heated at 143° F. for 30 min.) by the addition of solids-not-fat and calcium chloride. Calcium chloride concentrations as high as 0.08% gave no detectable off-flavor to the heated skimmilk.

The curd tension of milk has been the subject of numerous investigations, with the expressed purpose of obtaining a milk with less curd tension. This type of milk was considered desirable from a dietary standpoint (3). Decreases in curd tension have been noted as a result of heat treatment (1), the use of pancreatic enzymes (2), dilution with water (3), homogenization (10), and the addition of sodium salts (11). Hill (7) demonstrated that milk from Jersey cows generally had a higher curd tension than milk from Holstein cows. Weisburg *et al.* (12) found that naturally soft-curd milk contained a lower casein concentration and a lower concentration of calcium and phosphorus than did hard-curd milks. They showed that normal variations in the pH and salt balance had no effect on the curd tension value.

The purpose of this study was to determine the effects of various heat treatments on the curd tension of fluid skimmilk and to assess the ability of fortification with nonfat dry milk and calcium chloride to overcome these effects.

## EXPERIMENTAL PROCEDURES

Skimmilk was obtained by separating mixed whole milk from the college dairy herd with a cold milk separator. It was stored at 40 ± 2° F. until used. The skimmilk had a solids-not-fat content ranging from 8.8 to 9.1% and a butterfat content of 0.02 to 0.03%. Whenever the solids-not-fat content of the skimmilk was below 9.0%, it was adjusted to 9.0% by fortification with nonfat dry milk to serve as the control milk referred to in this paper. "Lo-Temp"[2] powder was used for fortification. Fortification was done at 95° F., whether before or after heating. Where used, calcium chloride (dihydrate crystal) was added to the heated skimmilk in the form of a 25% aqueous solution, to minimize localized precipitation.

All heat treatments were for 30 min. duration. Since the temperature of treatment was varied, specific temperatures will be given in the section on Results.

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[2] Trademark for a low-heat nonfat dry milk.

The American Dairy Science Association method (4) was used for curd tension determinations. Curd tension measurements were made on a Submarine Signal Company [3] curd tension meter.

## EXPERIMENTAL RESULTS

*Effect of solids-not-fat.* The first step in the study was to screen various high-temperature treatments with the curd tension test, to assess the ability of added nonfat dry milk to overcome the detrimental effects of the heat treatments on curd tension. It was assumed that if any high-temperature treatment lowered the curd tension of milk to such an extent that it could not be overcome by fortification, i.e., raised to a value comparable to curd tension of skimmilk containing 9% solids-not-fat and heated at 143° F. for 30 min., then it probably would not be worth while to study that heat treatment more closely at the present time.

[3] Submarine Signal Company, Boston, Massachusetts.

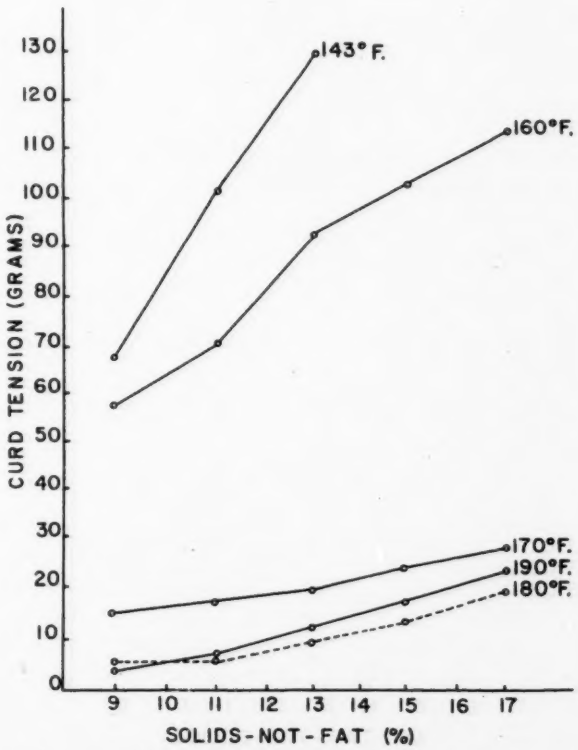


FIG. 1. Effect of heat treatment and solids-not-fat content on the curd tension of skimmilk. Fortification above 13% gave readings out of the range of the machine at 143° F.

The original skimmilk was divided into lots and fortified to give 11, 13, 15, and 17% total solids. These lots of milk were then heat-treated. The results are presented in Figure 1. Each point represents the average of three trials made on different days.

Perhaps the most significant effect shown is the decrease in the curd tension values of milk heated at 170° F. and above, as compared to milk heated at 160° F. for 30 min. Fortification to 17% total solids did not raise the curd tension of the milk heated at 170° F. and above to that of the original milk heated at 143° F.

From this work, it was decided to study heat treatments from 143 to 170° F. for 30 min. duration at closer temperature intervals. Temperature treatments of 143, 150, 155, 160, 165, and 170° F. were used. It was further decided to incorporate the effect of fortifying with nonfat dry milk both before and after heat treatment. In the first set of samples, the original milk was fortified to levels of 9, 10, 11, and 12% solids-not-fat before heat treatment. In the second set of samples, the original milk was heated at the desired temperature, cooled, and then fortified to levels of 9, 10, 11, and 12% solids-not-fat. Curd tension determinations were made on the fortified-heated and heated-fortified skimmilks after they had been stored for 0, 1, and 18 hr. at 40 ± 2° F. The effects of heat treatment, solid-not-fat content, and storage of 40° F. on the curd tension of milk fortified before and after heating, are presented in Tables 1 and 2. The values in both tables are averages of three trials made on different days with a different original skimmilk.

Both tables illustrate the inverse relationship of curd tension to the temperature of heat treatment. On the other hand, the curd tension increased as the

TABLE 1

Effects of heat treatment, solids-not-fat content, and storage on the curd tension of skimmilk fortified before heating

| Pasteurization temperature[a] | Hours storage | Curd tension | | | |
|---|---|---|---|---|---|
| | | Per cent solids-not-fat | | | |
| | | 9 | 10 | 11 | 12 |
| (° F.) | | (g.) | | | |
| 170 | 0 | 8.5 | 8.8 | 8.2 | 9.2 |
| | 1 | 7.5 | 7.6 | 7.6 | 8.0 |
| | 18 | 6.9 | 7.3 | 7.3 | 8.0 |
| 165 | 0 | 24.0 | 26.0 | 28.0 | 32.5 |
| | 1 | 19.5 | 23.5 | 25.0 | 27.0 |
| | 18 | 18.5 | 21.0 | 22.5 | 26.2 |
| 160 | 0 | 33.5 | 40.7 | 47.2 | 59.0 |
| | 1 | 30.2 | 39.0 | 44.2 | 54.7 |
| | 18 | 27.5 | 34.2 | 39.2 | 43.2 |
| 155 | 0 | 47.5 | 58.2 | 72.0 | 86.7 |
| | 1 | 46.5 | 63.2 | 72.0 | 88.7 |
| | 18 | 44.2 | 56.7 | 72.0 | 78.5 |
| 150 | 0 | 50.5 | 63.0 | 75.0 | 98.0 |
| | 1 | 48.2 | 63.0 | 76.2 | 88.0 |
| | 18 | 44.7 | 52.2 | 67.5 | 76.5 |
| 143 | 0 | 54.2 | 67.2 | 80.0 | 100.0 |
| | 1 | 51.0 | 64.2 | 79.2 | 91.2 |
| | 18 | 51.0 | 63.7 | 79.0 | 91.0 |

[a] 30-min. holding time.

TABLE 2

Effects of heat treatment, solids-not-fat content, and storage on the curd tension of skimmilk fortified after heating

| Pasteurization temperature[a] | Hours storage | Curd tension | | | |
|---|---|---|---|---|---|
| | | Per cent solids-not-fat | | | |
| | | 9 | 10 | 11 | 12 |
| (° F.) | | (g.) | | | |
| 170 | 0 | 9.6 | 12.0 | 16.6 | 25.0 |
| | 1 | 8.1 | 10.0 | 15.5 | 22.8 |
| | 18 | 8.1 | 10.1 | 15.0 | 22.3 |
| 165 | 0 | 21.0 | 25.0 | 35.0 | 43.5 |
| | 1 | 19.0 | 22.5 | 29.0 | 35.0 |
| | 18 | 17.2 | 21.7 | 27.0 | 31.0 |
| 160 | 0 | 34.7 | 42.2 | 52.5 | 66.2 |
| | 1 | 31.5 | 38.2 | 52.2 | 63.2 |
| | 18 | 28.2 | 35.0 | 44.5 | 56.5 |
| 155 | 0 | 48.2 | 59.5 | 75.7 | 88.0 |
| | 1 | 50.2 | 65.2 | 75.0 | 91.0 |
| | 18 | 44.2 | 59.0 | 66.2 | 82.0 |
| 150 | 0 | 53.0 | 68.5 | 78.0 | 95.0 |
| | 1 | 49.5 | 64.5 | 74.5 | 89.7 |
| | 18 | 45.5 | 60.0 | 70.2 | 81.5 |
| 143 | 0 | 53.5 | 65.5 | 79.5 | 99.7 |
| | 1 | 52.5 | 60.7 | 75.7 | 94.2 |
| | 18 | 51.7 | 60.7 | 74.7 | 93.5 |

[a] 30-min. holding time.

solids-not-fat content of the milk was increased. Although curd tension dropped when the skimmilk was held for 18 hr., the decrease was not nearly so great as that reported for the elasticity of rennet curd by Hansson and Hietaranta (5). They found that the elasticity (curd resiliency) dropped approximately 75% when the milk was stored at 40° F. for 24 hr.

An important difference between the data presented in the two tables is due to the effect on curd tension of adding nonfat dry milk before and after heat treatment. When milk was fortified before heat treatment (Table 1), that which contained 9% solids-not-fat and was heated at 143° F. for 30 min., had a curd tension of approximately 54 g.[4] at 0 hr. of storage. A comparable curd tension value, or higher, was obtained by heating milk containing 9, 10, 11, and 12% solids-not-fat at 155° F. and milk containing 12% solids-not-fat at 160° F. for 30 min.

More increase in curd tension due to increased increments of solids-not-fat is observed at the higher temperatures when the milk was fortified after heating (Table 2), than was found when the milk was fortified before heating (Table 1). This effect probably is due to the low-heat treatment to which the powder was subjected during its manufacture.

[4] It may be noted that the average curd tension of the original skimmilk in Figure 1 was approximately 70 g. That milk was tested in the fall, whereas the remaining samples were prepared from milk produced in the spring, when cows were on pasture. This probably accounts for the lower average curd tension values obtained. Since subsequent tests showed that acceptable cottage cheese could be made from milk (9% solids-not-fat heated at 143° F. for 30 min.) with a curd tension of approximately 54 g., this curd tension value was accepted as the standard.

There is very little difference in the curd tension at 0 hr. aging of milk of 9% solids-not-fat heated at 143 and 150° F. Comparable curd tension values (53 g.), or higher, are evident at 155° F. for milk containing 10, 11, and 12% solids-not-fat, and 160° F. for milk containing 11 and 12% solids-not-fat.

*Effect of calcium chloride.* The literature contains contradictory statements as to the effects of using calcium chloride to overcome the detrimental effects of heat treatment. The effects of calcium chloride on the curd tension of fresh skimmilk fortified to 9, 10, 11, and 12% solids-not-fat, both before and after heat treatments of 143, 150, 155, 160, 165, and 170° F., were determined. Calcium chloride (25% aqueous solution) was then added to the aliquots of milk, to give concentrations of 0.00, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.35% added calcium chloride. The mixture was shaken three different times during a 15-min. period before its curd tension was determined.

Figure 2 shows typical results obtained when varying levels of calcium chloride were added to milk heated at temperatures ranging from 143 to 170° F. In all cases, there was a maximum curd tension obtained with serial additions of calcium chloride, the maximum occurring at different levels for different

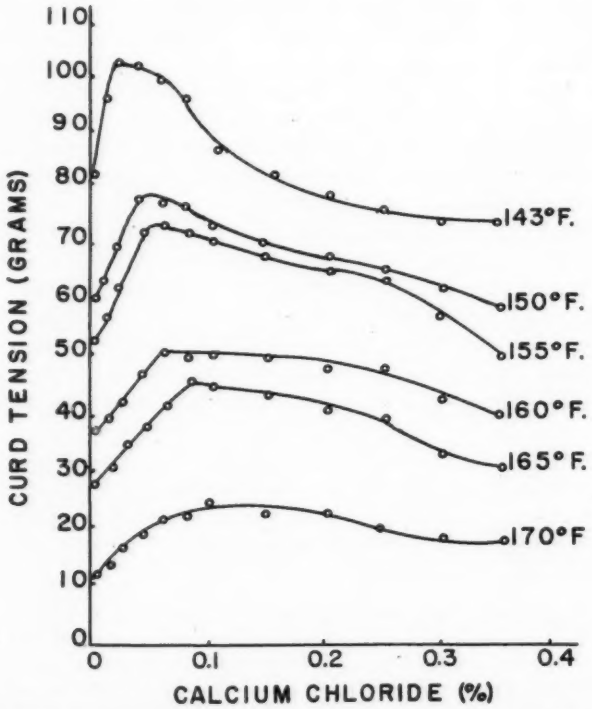


FIG. 2. The effect of calcium chloride on the curd tension of milk fortified before heating to 12% solids-not-fat and heated at the specified temperatures for 30 min.

treatments. The maximum curd tension values for all solids levels and heat treatments studied are reported in Tables 3 and 4. The curd tension values for milk of all solids levels heated at 160° F. are high in relation to the pattern set forth by the other heat treatments. As can be observed from the standard deviations associated with the average curd tension values, there was considerably more variability associated with the curd tension data at 160° F. than at the other temperatures. No explanation for this is evident.

Table 3 illustrates the direct relationship between heat treatment and the amount of calcium chloride required to produce a maximum curd tension in milk fortified before heating. As the heat treatment was increased, the amount of calcium chloride required also increased. For milk heated at 143° F. for 30 min., approximately 0.02% calcium chloride was necessary to produce a maximum curd tension, whereas 0.10% was necessary in milk heated at 170° F. for 30 min. However, the amount of calcium chloride required to produce a maximum curd tension remained the same for milk at a given heat treatment with varying solids-not-fat levels.

Table 4 shows that the amount of calcium chloride necessary to produce a maximum curd tension in milk fortified after heating is approximately the same as for milk fortified before heating (Table 3), and again varied directly with heat treatment but remained the same for milk with varying solids-not-fat content at a given heat treatment.

TABLE 3

Maximum curd tension obtained by the addition of calcium chloride to milk fortified before heating

| Pasteurization temperature[a] | Solids-not-fat | $CaCl_2$ giving maximum curd tension | Maximum curd tension | Variation in curd tension |
|---|---|---|---|---|
| *(° F.)* | *(%)* | *(%)* | *(g.)* | *(standard deviation)* |
| 170 | 9 | | | |
| | 10 | | | |
| | 11 | | | |
| | 12 | 0.10 | 35.0 | 2.36 |
| 165 | 9 | | | |
| | 10 | | | |
| | 11 | 0.08–.10 | 37.3 | 2.95 |
| | 12 | 0.08–.10 | 44.3 | 1.77 |
| 160 | 9 | 0.08 | 52.0 | 8.28 |
| | 10 | 0.08 | 62.6 | 10.65 |
| | 11 | 0.08–.10 | 76.0 | 8.86 |
| | 12 | 0.08–.10 | 81.6 | 7.68 |
| 155 | 9 | 0.06–.08 | 45.6 | 1.18 |
| | 10 | 0.06–.08 | 56.6 | 1.18 |
| | 11 | 0.06–.08 | 66.6 | 1.77 |
| | 12 | 0.06–.08 | 72.3 | 2.36 |
| 150 | 9 | 0.04–.06 | 53.6 | 1.18 |
| | 10 | 0.04–.06 | 56.0 | 2.36 |
| | 11 | 0.04–.06 | 66.3 | 1.77 |
| | 12 | 0.04–.06 | 77.6 | 2.96 |
| 143 | 9 | 0.02–.04 | 65.6 | 0.59 |
| | 10 | 0.02–.04 | 69.3 | 1.77 |
| | 11 | 0.02 | 81.0 | 1.18 |
| | 12 | 0.02–.04 | 101.6 | 2.96 |

[a] 30-min. holding time.

TABLE 4

Maximum curd tension obtained by the addition of calcium chloride to milk fortified after heating

| Pasteurization temperature[a] | Solids-not-fat | $CaCl_2$ giving maximum curd tension | Maximum curd tension | Variation in curd tension |
|---|---|---|---|---|
| *(° F.)* | *(%)* | *(%)* | *(g.)* | *(standard deviation)* |
| 170 | 9 | | | |
| | 10 | | | |
| | 11 | 0.10 | 32.6 | 2.95 |
| | 12 | 0.10 | 40.6 | 1.18 |
| 165 | 9 | | | |
| | 10 | 0.08–.10 | 41.6 | 1.77 |
| | 11 | 0.08–.10 | 56.6 | 1.77 |
| | 12 | 0.08–.10 | 63.6 | 1.18 |
| 160 | 9 | 0.08 | 52.3 | 4.71 |
| | 10 | 0.08 | 65.6 | 7.11 |
| | 11 | 0.08 | 79.3 | 10.04 |
| | 12 | 0.08 | 89.0 | 5.32 |
| 155 | 9 | 0.06–.08 | 44.6 | 2.95 |
| | 10 | 0.06–.08 | 58.0 | 3.55 |
| | 11 | 0.06–.08 | 71.0 | 1.18 |
| | 12 | 0.06–.08 | 72.3 | 6.50 |
| 150 | 9 | 0.04–.06 | 53.3 | 2.96 |
| | 10 | 0.04–.06 | 65.3 | 2.96 |
| | 11 | 0.04–.06 | 77.3 | 3.55 |
| | 12 | 0.04–.06 | 95.0 | 2.36 |
| 143 | 9 | 0.02–.04 | 67.3 | 2.36 |
| | 10 | 0.02–.04 | 73.0 | 1.18 |
| | 11 | 0.02–.04 | 84.6 | 1.18 |
| | 12 | 0.01–.04 | 108.6 | 9.45 |

[a] 30-min. holding time.

The data presented in Tables 3 and 4 indicate that calcium chloride can be utilized to help overcome the detrimental effects of heat treatment on curd tension. Milk fortified to 11 and 12% solids-not-fat and heated at 160° F. for 30 min., produced a curd tension value well above 54 g. when calcium chloride was used. For milk fortified after heating to 11 and 12% solids-not-fat, the use of optimum amounts of calcium chloride permitted heating as high as 165° F. for 30 min. without reducing the curd tension below 54 g.

Three judges tasting independently were able to detect a slight salty flavor in the milk at the highest concentration of calcium chloride reported in this paper (0.10%), but were not able to detect any off-flavor at a lower concentration attributable to the addition of calcium chloride.

## DISCUSSION

It has been confirmed that the curd tension of skimmilk that had been heated excessively could be restored by increasing the concentration of soilds-not-fat and by adding calcium chloride. This was expected, since it is well known that increasing concentrations of casein and calcium favor strong curd character. The primary objective of this paper was to determine the maximum heat treatment to which skimmilk could be subjected and its original curd tension subsequently restored by the addition of solids-not-fat and calcium chloride. Milk

containing 9% solids-not-fat and heated to 143° F. for 30 min. had a curd tension value of 54 g. and was taken as the standard.

When the solids-not-fat level was raised to 10% or above, it was immaterial whether fortification was done before or after heat treatment at 150 and 155° F., because the benefits of fortification on curd tension exceeded the detrimental effects of heat.

More calcium was needed to produce a maximum curd tension as the heat treatment was increased. This could be partially explained as a restoration of precipitated calcium salts (6), or on the basis that the milk did not contain the optimum amount of calcium for maximum curd formation.

The fact that skimmilk can be heated to temperatures at which the curd tension can not be restored by added solids-not-fat and calcium, might indicate that denatured serum proteins interfere physically with normal curd formation or form complexes that retard or reduce curd strength. Rudnick (8) favored the former explanation.

The maximum heat treatment to which milk was subjected and the curd tension restored to approximately its original value (54 g.) was 165° F. for 30 min.

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# EFFECT OF HARD WATER ON THE HEAT STABILITY OF SKIMMILK POWDER[1]

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**SUMMARY**

Protein stability in reconstituted skimmilk is adversely affected by high calcium and magnesium levels in the reconstituting water. The heat stability, as measured by the Storrs stability test, is a function of total hardness, and is not changed by varying the calcium-to-magnesium ratio. The effect of magnesium ions can be balanced by sequestration with equivalent quantities of sodium hexametaphosphate, but with calcium more than equivalent quantities of this sequestering agent are needed. Sequestration of a portion of the hardness of very hard waters is suggested as a possible method for avoiding premature protein coagulation during heat processes used in the manufacture of cottage cheese and in the preparation of media for propagation of cheese starters.

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When very hard water is used to reconstitute skimmilk powder, premature coagulation of proteins in the milk occurs during certain heat treatments. In some areas in the Province of Ontario, where the hardness in water exceeds 1,000 p.p.m., this problem has been encountered. Figure 1 shows a typical example of the effect of one heat treatment on milk reconstituted with water. Preliminary experiments indicated that the heat stability of the dry milk is inversely proportional to the degree of hardness in the reconstituting water; therefore, the effects of calcium and magnesium ions on heat stability were investigated, both separately and together, using the Storrs heat stability test as an index of stability.

## PREPARATION OF SYNTHETIC HARD WATER SOLUTIONS

Stock calcium and magnesium solutions were prepared from appropriate quantities of calcium chloride, magnesium chloride, and magnesium sulphate. Four series of synthetic hard waters, each including hardness levels of 0, 50, 100, 200, 400, and 800 p.p.m. (calculated as $CaCO_3$) were made by diluting the stock solutions with distilled water. One series contained only calcium, one contained only magnesium, and the other two contained both calcium and magnesium in the ratios 2:1 and 1:6. The latter ratio was selected to obtain a synthetic hard water in which the magnesium ion content was much in excess of the calcium ion content. The hardness levels of these solutions were checked by the method of Betz and Noll (1), using Eriochrome Black T as a total hardness indicator, and Murexide as a calcium indicator.

To determine the effect of sodium hexametaphosphate on the destabilizing influence of calcium and magnesium ion, synthetic hardness solutions were made up by mixing the stock hardness solutions with stock hexametaphosphate solution and distilled water. The calcium and magnesium equivalence of the hexameta-

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[1] This is the legal name of this product in Canada.

FIG. 1. The heat coagulation (200° F. for 1 hr.) of skimmilk powder reconstituted in extremely hard natural water obtained from a local cheese factory.

phosphate solution was determined by using it in place of EDTA in Betz and Noll titrations of the calcium and magnesium stock solutions. Sequestration levels of 25, 50, 75, and 100% indicate the respective percentage of an equivalent quantity of hexametaphosphate that was used in preparation of the synthetic hard waters.

## EXPERIMENTAL PROCEDURES AND RESULTS

Reconstituted skimmilk samples were prepared by mixing 24.72% g. of a low heat powder with 200 ml. of synthetic hard water, using a Waring Blender operated at high speed for 10 min. The milk samples thus prepared (about

11% total solids) were then stored for 12 hr. at 48° F., after which they were tested for heat stability by the method of Storrs (2).

A comparison of the Storrs stability numbers of milks reconstituted from two different low heat powders (Samples 1 and 2) at various hardness levels, but with no sequestering agent added, is shown in Figure 2. The Storrs stability

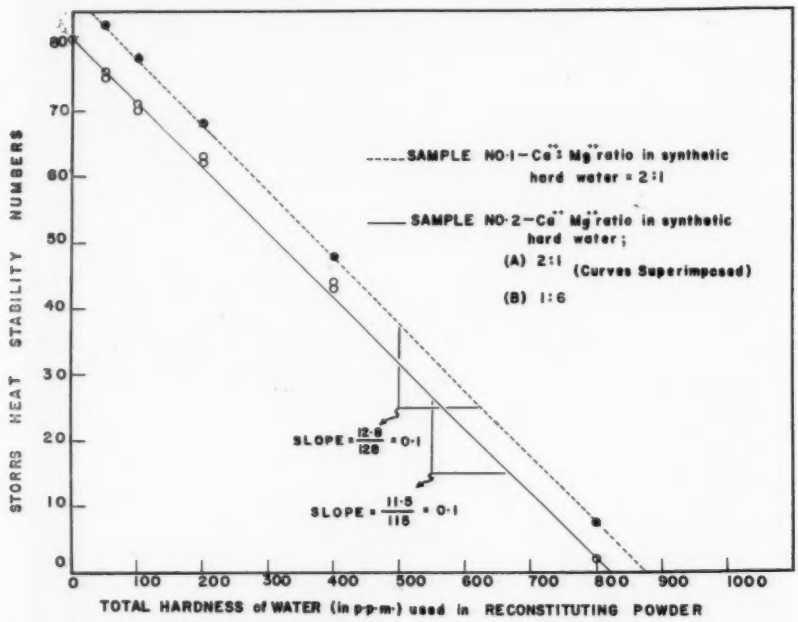


FIG. 2. The Storrs heat stability test on two different samples of low heat skimmilk powder, showing the effect of the $Ca^{++}$ to $Mg^{++}$ ion ratio on the heat stability of the milk.

number was inversely proportional to the degree of total hardness of the reconstituting water. Hardness levels of 800 to 900 p.p.m. reduced the stability number to zero. The two powders produced parallel curves. When the calcium to magnesium ratio was changed from 2:1 to 1:6 with Powder Sample No. 2 (Figure 2), and when magnesium (zero per cent sequestration curve in Figure 3) or calcium (zero per cent sequestration curve in Figure 4) was used alone, identical curves were produced. This indicated that the destabilizing effects of calcium and magnesium ions are the same in the concentration range studied.

The addition of percentages of an equivalent quantity of sodium hexametaphosphate to magnesium hardness solutions produced the same percentage of stability recovery in the reconstituted milks (Figure 3). When 100% of the equivalent quantity of sequestering agent was added, the resultant reconstituted milks had the same stability number as milk reconstituted with distilled water.

The addition of percentages of an equivalent quantity of sodium hexameta-

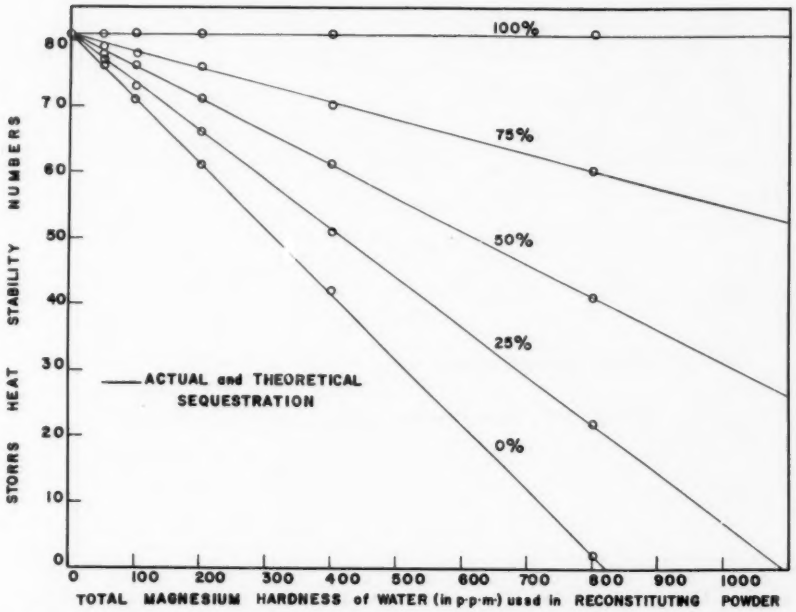


FIG. 3. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for $Mg^{++}$ ions.

phosphate to calcium hardness solutions resulted in less than the theoretical recovery (Figure 4). Thirty-three per cent more than an equivalent quantity of hexametaphosphate was required for complete stability recovery, and in the 800 p.p.m. hardness solution this quantity of sequestering agent failed to provide as much recovery as that achieved by the addition of only 25% of an equivalent amount.

The stability recovery achieved by addition of hexametaphosphate to hard water solutions containing both calcium and magnesium is shown in Figure 4. As would be expected, the percentage stability recovery here also is less than the percentage of an equivalent quantity of added hexametaphosphate.

An attempt was made to protect reconstituted milk made with synthetic hard water against destabilization during each of two heat treatments commonly used in the preparation of cultures for the manufacture of cottage cheese. The heat treatments used were 180° F. for 1 hr. and 245° F. for 15 min. Milks reconstituted from 800 p.p.m. hardness water containing various percentages of an equivalent quantity of hexametaphosphate were subjected to the heat treatments and were then examined for evidence of coagulation. An additional set of samples containing EDTA in place of hexametaphosphate was treated in the same way. Milk samples containing no sequestering agent coagulated during both heat treatments. The addition of EDTA equivalent to 25% or more of

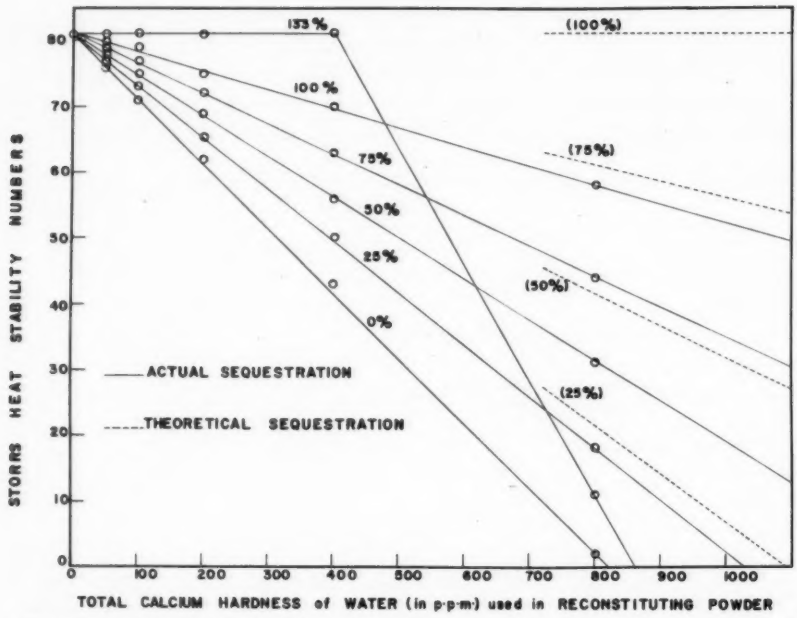


FIG. 4. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for $Ca^{++}$ ions.

800 p.p.m. of hardness prevented coagulation during both heat treatments. The addition of hexametaphosphate equivalent to 25% or more of the 800 p.p.m. of hardness protected the milk against coagulation during the 180° F. hour treatment, but provided no protection against the 245° F.–15 min. treatment.

## DISCUSSION

The Storrs stability data shown in Figures 2, 3, 4, and 5 indicate that the heat instability of reconstituted powders is caused by the presence of excess calcium or magnesium ions. It is reasonable to expect that the stability of milk in which agents are used to sequester these excess ions would depend upon the heat stability of the complexes formed. The calcium and magnesium complexes formed by EDTA are known to be more heat stable than those formed by hexametaphosphate. The relative stabilities of these complexes are illustrated by the results of attempts to prevent coagulation during sterilizing treatments. Skimmilk powder reconstituted in hard water treated with sodium hexametaphosphate proved to be unstable when subjected to a laboratory sterilizing process (245° F. for 15 min.); whereas, the same powder reconstituted in water treated with EDTA showed no instability during the same heat treatment. Both reconstituted powders were stable when subjected to the less severe plant sterilizing treatment (180° F. for 1 hr.).

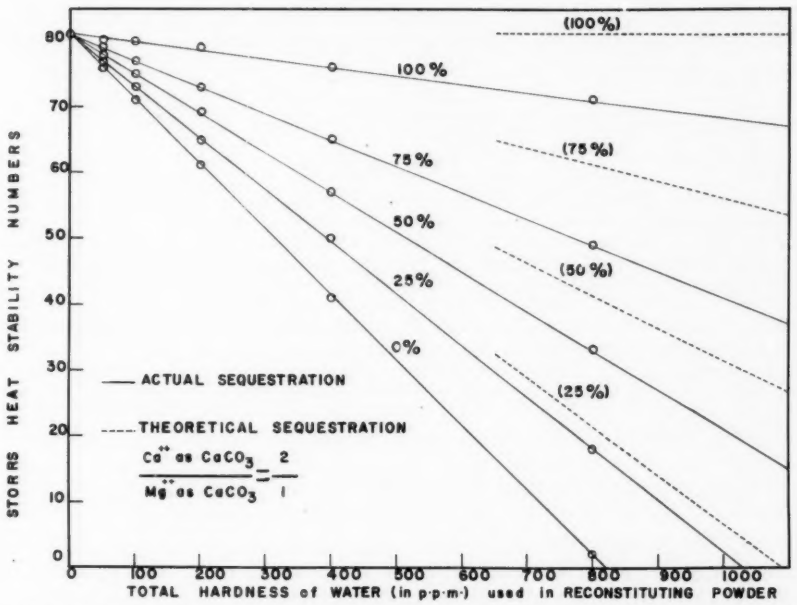


FIG. 5. The Storrs heat stability test on skimmilk powder (Sample No. 2) reconstituted in water treated with sodium hexametaphosphate as a sequestering agent for $Ca^{++}$ and $Mg^{++}$ ions.

The experimental results indicate that the stability of a reconstituted powder may be improved in some heat treatments by the addition of sodium hexametaphosphate to the water used in reconstitution. By determining the Storrs heat stability number of the skimmilk powder in question, and the hardness at which instability of the reconstituted milk occurs, subsequent calculation may be made of the minimum amount of sodium hexametaphosphate required to prevent the heat coagulation of the milk in the existing heat treatment.

Before employing sodium hexametaphosphate to prevent the heat coagulation of reconstituted milk, it would be advisable to consult local health authorities on the legality of its use.

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# FACTORS INFLUENCING THE METABOLIC ACTIVITY OF BOVINE SPERMATOZOA. V. SEASON

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**SUMMARY**

The aerobic metabolic activity (oxygen consumption, fructose utilization, and lactic acid accumulation) of semen and washed spermatozoa from collections made from eight bulls during two seasons (winter and summer) and 2 yr. was determined in two diluents and three treatments. The treatments included the suspension of washed cells in diluent containing fructose or diluted seminal plasma from the same ejaculate or that from the same bull stored from the previous season. Summer-collected cells lived through the 4-hr. incubation at 37° C. better, consumed more oxygen, and accumulated less lactic acid whether incubated as semen or as washed cells resuspended in fructose or in their own diluted seminal plasma. Diluted seminal plasma of the previous season reversed some of these trends. The primary though not exclusive seasonal effect in metabolism resides in the cells.

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In many of the vertebrates, spermatogenesis is a cyclic phenomenon. Although spermatozoa are produced throughout the year in the bull, some seasonal fluctuations in spermatogenesis and fertility exist. For example, Erb *et al.* (4) found in the Purdue herd that the highest conception rate occurred in May, and the lowest in August; while in Louisana, the conception rate was reported by Seath and Staples (11) to be lower during the summer months than in any other period of the year.

Evidence indicates that temperature, light, relative humidity, and possibly other atmospheric factors influence the quality of the semen produced by the bull (3). Under controlled conditions, the continuous exposure of young dairy bulls to environmental temperatures exceeding 85° F. for periods longer than 5 wk. was reported to impair spermatogenesis (2). Other studies have indicated that light also may be an important factor. Mercier and Salisbury (6, 7) studied the conception rate of naturally bred cattle in Eastern Canada and the artificially bred cattle of New York State. Temperature changes at that latitude did not measurably affect the fertility level of the cattle. However, the length of daylight was directly related to the conception rate.

If part of the variations in fertility which occur with the seasons of the year can be attributed to the male, it is reasonable to expect that changes in biochemical behavior of the male reproductive cells might accompany the observed variations. Smith *et al.* (12) observed a significant stimulation of the succinate dehydrogenase activity, on washing, of bovine spermatozoa obtained during the winter and spring months. On the other hand, no significant activation of the succinate dehydrogenase occurred on washing of spermatozoa obtained during the summer and fall months.

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In an earlier examination of data obtained in this laboratory, the aerobic fructolysis of bovine semen collected during the winter was significantly greater than that collected in the summer months (10). Since this evidence was obtained from studies having other objectives, an experiment was designed to clarify the observation. In addition, the study was designed to determine whether the metabolic responses were due to the cells being different from season to season or to a difference in the seminal plasma.

## EXPERIMENTAL PROCEDURE

Semen specimens were collected from eight bulls during the winter and summer seasons of 2 yr. To represent the extremes in environmental conditions of temperatures and daylight hours, ejaculates were collected during the months of December to February for the period designated as winter, and during July and August for the summer. The mean maximum and minimum temperatures for the first and second winters were 30.8 and 13.9° F. and 36.7 and 20.4° F.; the daylight hours were 10.2 and 9.5 for the periods of collection during each of the two winters. The mean maximum and minimum temperatures for the two summers were 88.9 and 67.5° F. and 81.5 and 62.4° F.; the daylight hours were 14.9 and 14.9, respectively.

Two ejaculates were collected from each of eight bulls at intervals of two to eight days during the winter of 1954-55, summer of 1955, winter of 1955-56, and summer of 1956, making a total of 64 ejaculates. Aliquot specimens were suspended in two diluents, saline and phosphate buffer, which were subjected to three treatments: untreated diluted semen, twice-washed spermatozoa suspended in fructose diluent, and twice-washed spermatozoa suspended in diluted seminal plasma from the same ejaculate. Forty-eight of these 64 ejaculates were subjected to an additional treatment, i.e., specimens of the twice-washed cells were resuspended also in diluted (saline and phosphate buffer) seminal plasma obtained during the preceding season. Thus, there was a total of 480 observations in the entire experiment.

Semen specimens and diluents, physiological saline and sodium phosphate buffer, were prepared in the manner described previously (10). Briefly, each semen sample was divided into three portions, A, B, and C. Portion A remained untreated, B was washed twice with phosphate buffer (pH 7) and aliquot specimens were suspended in phosphate-fructose solution, seminal plasma (which consisted of 0.6 ml. phosphate buffer and 0.2 ml. seminal plasma that was removed prior to the washing of the cells), and seminal plasma (in similar proportions) from the same bull of the previous season (stored at 0° F.). Portion C was treated as in B, but the sodium phosphate buffer solution was replaced with physiological saline (0.9% NaCl).

Spermatozoa suspended in a medium free from seminal plasma were obtained by centrifuging whole semen at approximately 400 × gravity for 10 min. and removing the supernatant seminal plasma. The sperm cells were resuspended in the diluting solution to the original volume of the ejaculate. The suspension was again centrifuged, the supernatant removed, and the cells resuspended in fresh diluent. The process was repeated a second time.

In subsequent dilutions of the cells, physiological saline was added to those cells washed with saline, and phosphate buffer was added to the phosphate-washed cells.

Respiration was measured by the direct method of Warburg (13) at 37° C. for 4 hr. in 5-ml. single-side-arm reaction flasks. Carbon dioxide was absorbed with 20% KOH (0.1 ml.) placed in the center well. Two-tenths of a milliliter of semen or spermatozoa suspension containing approximately $3 \times 10^8$ cells was placed in the side arm and added to 0.8 ml. of diluent in the main compartment after an equilibration period of 10 min. Aliquot specimens were taken in the beginning and end of each experiment for fructose (5, 9) and lactic acid (1) determinations.

When the study was initiated, seminal plasma of semen collected during the seasonal period preceding the beginning of the study was not available. Thus, three seasons (one winter and two summers) were represented for the treatment involving the resuspension of twice-washed spermatozoa in seminal plasma of the preceding season.

The bulls were fed hay and University Grain Mix 38 and were allowed to exercise daily in a dry lot, but were not pastured.

## RESULTS

*Treatment I—diluted whole semen.* The summary of the average aerobic metabolic responses of diluted semen collected during the two summers and two winters is given in Table 1. The percentage of motile cells immobilized during the 4-hr. incubation period in saline was lower than it was in phosphate buffer. The respiratory activity of semen diluted in physiological saline was approximately twice as great as that diluted in phosphate buffer. Fructose uptake and lactic acid accumulation were generally lower in saline diluent than in phosphate buffer. These responses were consistent throughout the study.

The percentage of motile cells immobilized during the incubation period was slightly lower for the summer-collected cells than for the winter-collected cells. When the seasonal fructolytic responses were compared, winter-collected semen respired less but utilized more fructose and accumulated more lactic acid

TABLE 1

Summary of the aerobic metabolism of semen

| Diluent | Seasons of collection | Motility | | | | Aerobic activity | | | $+Z_{lactate}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | Initial | | Final | | $-Zo_2$ | $-Z_{fructose}$ | $+Z_{lactate}$ | $-Z_{fructose}$ |
| | | % | Rate | % | Rate | µl. | µg. | µg. | % |
| NaCl | Winter | 53.2 | 3.2 | 40.5 | 2.2 | 9.1 | 42.4 | 17.9 | 42 |
| | Summer | 61.0 | 3.4 | 48.2 | 2.7 | 9.9 | 35.6 | 6.4 | 18 |
| $HPO_4$ | Winter | 52.8 | 3.2 | 24.1 | 1.1 | 5.0 | 47.0 | 27.3 | 58 |
| | Summer | 61.0 | 3.4 | 35.4 | 2.0 | 5.2 | 35.2 | 24.4 | 69 |

Mean of 32 observations.
Mean cell count: Winter, $263.6 \times 10^6$ cells/ml of diluent.
Summer, $285.5 \times 10^6$ cells/ml of diluent.

than summer-collected semen. Though the differences were not statistically significant, the seasonal differences with these unwashed spermatozoa in semen were similar to the earlier results (8) under the same conditions.

The individual seasonal metabolic responses of semen are shown in Table 2. The mean initial motility of the semen samples of the second winter was approximately 9% higher than that of the samples collected during the first winter, although greater fructose uptakes were encountered with the semen of the first winter. Differences in the metabolic activity of the two winters were not significant.

TABLE 2

Seasonal aerobic metabolic activity of semen

| Seasons of collections | Diluent | Motility | | | | Aerobic activity | | | $+Z_{lactate}$ |
|---|---|---|---|---|---|---|---|---|---|
| | | Initial | | Final | | $-Zo_2$ | $-Z_{fructose}$ | $+Z_{lactate}$ | $-Z_{fructose}$ |
| | | % | Rate | % | Rate | μl. | μg. | μg. | % |
| 1st winter | NaCl | 50.7 | 3.2 | 39.7 | 2.2 | 9.4 | 50.7 | 13.7 | 27 |
| | $HPO_4$ | | | 24.1 | 1.2 | 4.9 | 55.0 | 31.3 | 57 |
| 2nd winter | NaCl | 55.6 | 3.2 | 41.3 | 2.2 | 8.8 | 34.2 | 22.1 | 65 |
| | $HPO_4$ | | | 24.4 | 1.0 | 5.2 | 38.9 | 23.3 | 60 |
| 1st summer | NaCl | 56.9 | 3.4 | 43.8 | 2.8 | 10.1 | 28.9 | 1.7 | 6 |
| | $HPO_4$ | | | 30.1 | 2.1 | 5.6 | 29.7 | 23.9 | 80 |
| 2nd summer | NaCl | 65.0 | 3.5 | 52.5 | 2.6 | 9.7 | 42.4 | 11.2 | 26 |
| | $HPO_4$ | | | 40.6 | 1.8 | 4.8 | 40.8 | 24.8 | 61 |

Mean of 16 observations.
Mean cell count: 1st winter, $272.6 \times 10^6$ cells/ml of diluent
2nd winter, $254.7 \times 10^6$ cells/ml of diluent.
1st summer, $305.0 \times 10^6$ cells/ml of diluent.
2nd summer, $266.0 \times 10^6$ cells/ml of diluent.

In comparing the summer seasons, the mean initial motility was approximately 12% higher for the semen samples collected in the second than in the first summer, and the fructose uptake and lactic acid accumulations were higher for the second summer. However, the degree of variation in respiration, substrate uptake, and lactate accumulation was large, and the differences noted between the two summers were not significant.

*Treatment II—twice-washed spermatozoa diluted in fructose solution.* In the phase of the experiment just discussed (Treatment I), the fructolytic activity of winter-collected semen was greater than that of summer-collected semen. However, when spermatozoa from these same ejaculates were washed and resuspended in fructose diluent, summer-collected spermatozoa were more active metabolically than winter-collected spermatozoa.

About 58% of all the winter-collected cells were immobile after the 4-hr. incubation period, and 37% for the summer-collected spermatozoa (Table 3). Respiration and fructose uptake of summer-collected spermatozoa were significantly ($P < 0.01$) greater than winter-collected cells. Lactic acid accumulation, though greater in the summer-collected cells, was not significantly ($P > 0.05$) different. These responses of washed cells resuspended in fructose diluent differed from the aerobic glycolysis of spermatozoa in semen. The differences in the aerobic gly-

TABLE 3

Aerobic metabolic behavior in fructose solution of twice-washed spermatozoa

| Seasons of collection | Diluent | Mean cell count $\times 10^6$ | Motility | | | | Aerobic activity | | | $+Z_{lactate}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | | Final | | $-Zo_2$ | $-Z_{fructose}$ | $+Z_{lactate}$ | $-Z_{fructose}$ |
| | | | % | Rate | % | Rate | µl. | µg. | µg. | % |
| Winter | NaCl | 254.4 | 48.2 | 2.8 | 31.2 | 1.4 | 8.2 | 19.8 | 6.0 | 30 |
| | $HPO_4$ | 248.8 | 42.7 | 2.4 | 6.8 | 0.4 | 4.1 | 18.8 | 17.1 | 91 |
| Summer | NaCl | 359.9 | 52.8 | 3.1 | 39.0 | 2.0 | 9.0 | 22.0 | 6.8 | 31 |
| | $HPO_4$ | 344.4 | 50.0 | 2.8 | 25.9 | 1.2 | 4.7 | 25.0 | 19.5 | 78 |

colysis of semen and washed cells resuspended in fructose diluent suggest that summer-collected semen was inherently as active in their fructolytic processes as winter-collected semen. The difference in behavior of these two seasonally grouped cells may be due either to the seminal plasma or to the removal of a component or components from the cells by washing.

*Treatment III—twice-washed spermatozoa resuspended in the plasma of the same ejaculate.* The responses of twice-washed spermatozoa resuspended in diluted seminal plasma of the same ejaculate are summarized in Table 4. The

TABLE 4

Effect of diluent and seminal plasma (plasma of the same ejaculate) on twice-washed spermatozoa

| Seasons of collection | Diluent | Mean cell count $\times 10^6$ | Motility | | | | Aerobic activity | | | $+Z_{lactate}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | | Final | | $-Zo_2$ | $-Z_{fructose}$ | $+Z_{lactate}$ | $-Z_{fructose}$ |
| | | | % | Rate | % | Rate | µl. | µg. | µg. | % |
| Winter | NaCl | 251.2 | 48.4 | 2.8 | 42.6 | 2.4 | 9.6 | 27.8 | 19.4 | 70 |
| | $HPO_4$ | 241.8 | 42.5 | 2.4 | 26.1 | 1.2 | 5.6 | 23.5 | 41.6 | 177 |
| Summer | NaCl | 359.9 | 52.8 | 3.1 | 50.4 | 3.0 | 9.9 | 28.8 | 16.6 | 58 |
| | $HPO_4$ | 344.4 | 50.0 | 3.0 | 40.0 | 2.3 | 5.6 | 32.2 | 31.4 | 98 |

decrease in motility of winter- and summer-collected cells during the test period was about 24 and 10%, respectively. Respiration of winter- and summer-collected cells was approximately the same and was not statistically ($P > 0.05$) different. The fructose loss in summer-collected samples was approximately 16% more than in winter-collected spermatozoa, but the difference was not significant. On the other hand, the lactic acid accumulated in summer-collected samples was significantly ($P < 0.01$) lower than in winter cells. This lower accumulation of lactate by summer-collected, twice-washed spermatozoa is similar to that observed in semen, but contrary to that observed in twice-washed cells provided with fructose as a sole substrate. This result suggests that some entity, which may have been present or absent in the seminal plasma obtained during the summer months, depressed the lactic acid accumulation of twice-washed, summer-collected cells.

*Treatment IV—twice-washed spermatozoa resuspended in the plasma collected during the preceding season.* To test whether seminal plasma influenced the seasonal metabolic pattern of bovine spermatozoa, cells from a bull, collected

during one season, were washed and resuspended in seminal plasma removed from semen collected in the preceding season from the same animal and frozen until used. Seminal plasma was not available from the summer season preceding the first winter of the study, but the data of the three subsequent seasons are presented in Table 5.

The percentage of winter-collected cells suspended in winter plasma (plasma of the same ejaculate) immobile at the end of the incubation period was approximately 24%, and winter-collected cells in summer plasma (plasma of the same bull but of the preceding season, or stored plasma) was 33%. The percentage of summer-collected washed cells suspended in summer plasma (plasma of the same ejaculate) immobilized during the incubation period was approximately 12%, and of summer-collected cells suspended in winter (stored) plasma, 18%.

The respiration, fructose uptake, and lactic acid accumulation (Tables 5 and 6) were not significantly different ($P > 0.05$) when spermatozoa collected in winter were twice-washed and resuspended in winter plasma (plasma of the same ejaculate), or in summer (stored) plasma. When summer cells washed and suspended in winter (stored) plasma were compared to those suspended in summer plasma (plasma of the same ejaculate), the fructose uptake was significantly ($P < 0.05$) greater. Respiration and lactic acid accumulation did not differ stastistically. When the activity of the cells collected in winter was compared to that of summer samples, regardless of the treatment of diluents, fructose uptake ($P < 0.01$) and lactic acid accumulation ($P < 0.05$) were greater in the summer cells.

## DISCUSSION

As reported earlier (10), and irrespective of treatments, the livability of the spermatozoa suspended in saline was better at the end of the incubation period than of those diluted in the phosphate buffer. Respiration of saline-diluted sperm cells was approximately twice as great as it was in phosphate diluent, but fructose uptake and lactic acid accumulation were lower. These responses were generally consistent throughout this study.

In the seasonal comparisons of the survival of the spermatozoa, whether in diluted semen, washed cells suspended in fructose diluent, or washed cells suspended in diluted seminal plasma of the same ejaculate, the percentage of motile spermatozoa at the end of the incubation was consistently higher in the summer- than in the winter-collected cells. The respiratory activity of summer-collected semen was higher, but the fructose uptake and lactate accumulation were lower than in the winter-collected semen. Upon washing, however, the aerobic fructolysis of the summer-collected cells, whether suspended in fructose diluent or in diluted seminal plasma of the same ejaculate, was greater, though the differences were not statistically significant. An exception was the significantly ($P < 0.01$) lower lactate accumulation of twice-washed summer-collected cells suspended in seminal plasma of the same ejaculate. It was interesting to note that summer-collected semen contained a slightly higher fructose content than winter-collected semen, a mean of 7.90 and 5.14 mg/ml of semen, respectively. However, the difference was not statistically significant when the "t" test was applied.

TABLE 5

Comparison of diluent and seminal plasma on the aerobic metabolic behavior of washed bovine spermatozoa

| Treatment | Diluent | Mean cell count $\times 10^6$ | Motility | | | | Aerobic activity | | | $+Z_{lactate}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Initial | | Final | | $-Zo_2$ | $-Z_{fructose}$ | $+Z_{lactate}$ | $-Z_{fructose}$ |
| | | | % | Rate | % | Rate | μl. | μg. | μg. | % |
| Winter cells | | | | | | | | | | |
| Winter plasma | NaCl | 251.2 | 48.4 | 2.8 | 42.6 | 2.4 | 9.6 | 27.8 | 19.4 | 70 |
| | $HPO_4$ | 241.8 | 42.5 | 2.4 | 26.1 | 1.2 | 5.6 | 23.5 | 41.6 | 177 |
| Summer plasma [a] | NaCl | 243.8 | 49.4 | 2.6 | 37.5 | 2.1 | 9.9 | 25.0 | 10.0 | 40 |
| (From previous season) | $HPO_4$ | 249.2 | 40.0 | 2.2 | 22.2 | 0.8 | 5.3 | 23.0 | 27.9 | 121 |
| Summer cells | | | | | | | | | | |
| Winter plasma | NaCl | 359.9 | 52.8 | 3.1 | 48.3 | 2.9 | 9.2 | 36.0 | 22.4 | 62 |
| (From previous season) | $HPO_4$ | 344.4 | 50.0 | 3.0 | 35.6 | 1.8 | 5.6 | 43.0 | 37.4 | 87 |
| Summer plasma | NaCl | 359.9 | 52.8 | 3.1 | 50.4 | 3.0 | 9.9 | 28.8 | 16.6 | 58 |
| | $HPO_4$ | 344.4 | 50.0 | 3.0 | 40.0 | 2.3 | 5.6 | 32.2 | 31.4 | 98 |

[a] One season.

TABLE 6
Summary of the analysis of variance of twice-washed cells resuspended in plasma

| | Oxygen uptake | Fructose utilization | Lactic Acid accumulation |
|---|---|---|---|
| Bulls | 7.79** | 0.90 | 2.58* |
| Treatments | 11.08** | 2.56** | 4.09** |
| W C W P vs. W C S P | 0 | 0.10 | 0.06 |
| S C W P vs. S C S P | 0.59 | 4.24* | 2.71 |
| S C vs. W C | 0 | 8.30** | 5.74* |

W = winter; S = summer; C = cells; P = plasma.
* = significant ($P < 0.05$).
** = highly significant ($P < 0.01$).

Comparing the responses of twice-washed spermatozoa suspended in diluted plasma (plasma from the same ejaculate and plasma collected during the preceding season) to fructose-containing diluent, the motility at the end of the incubation period of washed cells resuspended in plasma was better than in the diluent containing fructose. The metabolic responses, respiration, fructose uptake, and lactic acid accumulation of these cells were also greater ($P < 0.01$). Twice-washed spermatozoa, whether collected in winter or summer, did not utilize as much substrate as semen. Apparently, subjecting the cells to the mechanical process of washing was injurious.

In the preliminary report of this study, winter-collected cells were recorded to utilize more fructose (8). On re-examination of the values, an error in calculation was noted. Corrected values are as follows: the fructolytic activity ($-Z$ fructose) of winter-collected semen diluted in saline and phosphate was 40.4 and 44.5; of $+Z$ lactate was 18.8 and 27.7, respectively. The $-Z$ fructose of winter-collected, twice-washed cells suspended in winter plasma (diluted plasma from the same ejaculate) diluted with saline and phosphate was 30.0 and 27.0, and in summer plasma (diluted plasma from the preceding season), 25.3 and 20.5. The $+Z$ lactate values for these treatments were 20.2, 40.1, 12.9, and 27.5, respectively. These corrected values are consistent with the final analysis, in that the uptake of fructose by summer- rather than winter-collected, twice-washed cells was greater.

In an experiment of this nature, the confounding of advancing age of the bulls and the passage of the four collection periods, two winters and two summers, over a period of 2 yr. could not be eliminated as a possible source of variance in the metabolic measures. However, comparisons of the metabolic responses of the cells collected in one season of the year with those collected in the same season of the second year indicated that the differences were not marked. Furthermore, it would be difficult to assign the cause of the metabolic differences, had they occurred, to the aging of the bull or to the differences in the climatic conditions of two different years. Since the observed metabolic responses of spermatozoa collected in the same seasons a year apart were not markedly different, it would indicate that the aging effect of the bulls played little, if any, part in the metabolic responses observed between summer- and winter-collected spermatozoa.

These experiments leave little hope that any considerable part of the fertility differences in cattle noted from season to season are associated with changes in the metabolic capacities of the spermatozoa tested immediately after ejaculation. While the relation of fertility and metabolic rate was not directly tested in these studies, the metabolic changes noted as due to seasons were small and required detailed experimental control to demonstrate. Thus, other factors influencing the metabolic rate of spermatozoa would overwhelm the minor seasonal effects in random assays from many bulls. What is even more pertinent is that, in general, the summer-collected cells lived better during the 4-hr. incubation; whereas, in the central part of Illinois the fertility level of cattle tends to be depressed during the hot summer months.

The cause or causes of the metabolic differences of spermatozoa encountered with the seasons of the year are not presently known, and further work is necessary to identify them.

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# FAT DIGESTIBILITY BY DAIRY CALVES[1]

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**SUMMARY**

The utilization of fat in milk replacers by the dairy calf was studied. When a milk replacer composed predominantly of dried skimmilk was supplemented with tallow, coconut fat, grease, or butter, in the unhomogenized state, the fat was poorly digested. However, the inclusion of crude soybean lecithin in the milk replacer improved the utilization of tallow, coconut fat, and grease. The digestibility of coconut fat was improved to a greater extent than was that of tallow or grease by the inclusion of lecithin in the milk replacer.

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Milk replacers containing homogenized tallow, lard, butter oil, or hydrogenated soybean oil have been shown to be equal, or nearly equal, to whole milk in promoting growth in the dairy calf. On the other hand, the inclusion of nonhydrogenated vegetable oils, such as soybean oil and cottonseed oil, have been shown to be detrimental to the health of calves (2, 7, 9, 10, 13, 14). The digestibility of certain fats by the dairy calf has been studied to a limited extent. Parrish *et al.* (16) and Noller *et al.* (15) found the apparent digestibility of the ether extract of whole milk to be between 96 and 98%. Blaxter and Wood (3) concluded that the ether extract of whole milk had an apparent digestibility of 96%. A recent report by Raven and Robinson (17) demonstrated that the digestibility of butterfat in spray-dried whole milk was 97% by 2-wk.-old calves. When liquid skimmilk containing homogenized palm oil was spray-dried, the digestibility of the fat was 92%. Wing (20) found that homogenized hydrogenated soybean oil was 92% digested by the dairy calf. Cunningham and Loosli (5) fed calves purified milk replacer diets containing either lard or hydrogenated coconut oil in the homogenized state. They found that coconut fat was 86.4, 89.7, 85.5, 88.6, and 71.9% digested during the second, fourth, sixth, eighth, and 11th wk. of age, respectively. Lard was found to be 72.6, 77.0, 92.5, and 93.7% digested during the second, fourth, sixth, and 11th wk., respectively. Blaxter and Wood (4) noted that lard and a small amount of other fatty material had an apparent digestion coefficient of 44.9% when fed in a nitrogen-free diet. However, the apparent digestibility of this fat was increased if the calories of the diet supplied by protein were increased. They postulated that fat digestibility plateaued at slightly over 90% when between 15 and 18% of the dietary calories were supplied in the form of protein.

In most studies of high-fat milk replacers, the fats have been homogenized with the diet. Little information is available on the value of different fats when mixed directly with the dried milk replacer and fed in the unhomogenized state. Kastelic *et al.* (11), while developing a purified diet for the calf, noted that a

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mixture of butter and soybean lecithin did not need to be homogenized to produce adequate growth. On the other hand, cottonseed oil had to be thoroughly homogenized with lecithin to be a successful dietary ingredient. Huff *et al.* (8) observed a loss of hair about the tail and inside of the thighs of calves fed unhomogenized hydrogenated cottonseed oil with glycerol monostearate as an emulsifying agent. This characteristic alopecia was not noted if the diet was homogenized with or without glycerol monostearate. Lambert *et al.* (12) noted that when calves were fed a milk replacer containing hydrogenated soybean oil, superior growth resulted if lecithin was included in the diet. The following study was initiated to obtain additional data on the utilization of several fats when fed to dairy calves.

## EXPERIMENTAL PROCEDURES

Experiments were designed to study the digestibility of the following unhomogenized fats by the calf: (1) tallow, (2) coconut fat, (3) sweet butter, (4) tallow plus crude soybean lecithin, (5) coconut fat plus crude soybean lecithin, (6) homogenized coconut fat and crude soybean lecithin, (7) choice white grease, and (8) choice white grease plus lecithin. Originally, the design included the study of homogenized tallow plus crude soybean lecithin. Sweet butter was substituted for this fat after one period because of difficulties that occurred while attempting to homogenize the tallow. In addition, the fat clumped badly after homogenization, making representative sampling impossible. The first experiment involving Diets 1-6 was a randomized block design, consisting of five blocks of six calves each obtained at a different time over 12 mo. The second experiment, involving Diets 7 and 8, was of a completely random design and conducted later than the first experiment.

The composition of the experimental milk replacer diets is given in Table 1. The basal mixture had the following composition: spray-dried skimmilk, 100; Aurofac D (5 g. of chlortetracycline per pound), 1.50; vitamin A (20,000 I.U. per gram.)[2], 0.25; irradiated yeast (9,000 U.S.P. units of vitamin D per gram),

[2] Nopcay 20, Type IV, generously supplied by Nopco Chemical Co., Harrison, N. J.

TABLE 1
Composition of the high-fat milk replacers used

| Diet No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Ingredients: | (lb.) | | | | | | | |
| Basal mixture | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 |
| Edible coconut fat[a] | | 20 | | | 18 | 18 | | |
| Bleachable fancy tallow[b] | 20 | | | 18 | | | | |
| Sweet butter[c] | | | 25 | | | | | |
| Choice white grease[b] | | | | | | | 20 | 18 |
| Crude soybean lecithin[d] | | | | 2 | 2 | 2 | | 2 |

[a] Stabilized with 10 g. B.H.T. per 100 lb.
[b] Stabilized with Tenox 2 (propylene glycol, B.H.A., and citric acid).
[c] Stabilized with 45 g. B.H.T. per 100 lb.
[d] Alcolec S—American Lecithin Co., Woodside, N. Y.

0.017. In earlier studies at this laboratory, this basal mixture has been observed to be 94% digested by 2-wk.-old calves when fed as a reconstituted milk replacer. The fats used in these studies had the following titers (solidification temperature of the saponified fatty acids; A.O.A.C. *Official Methods of Analysis*, 8th ed.): tallow, 42° C.; coconut fat, 23° C.; butter, 37° C.; grease, 39° C.

The fat that was to be added to the basal mixture was melted and lecithin, when it was included, was added to the melted fat. The melted blend was combined with the basal mixture and stored at barn temperature. At the time of feeding, these diets were reconstituted with water at a ratio of one to ten. The homogenate of Diet 6 was prepared in a Gaulin two-stage homogenizer with 500 and 2,000 lb. p.s.i. pressure at the first and second valves, respectively. The homogenate contained three parts water and one part fat. The particle size of the homogenate was about 2 $\gamma$. At the time of feeding, the basal mixture of Diet 6 was reconstituted with water and an appropriate amount of homogenate added. The diets for the second experiment were prepared in a similar manner.

The animals used in these experiments were male Holstein calves obtained from local dairymen or auctions and were approximately three days of age upon arrival at our laboratory. The animals received a diet of whole milk alone for several days after arrival. The milk replacers were gradually substituted for the whole milk, so that after seven days the calves received only 200 g. of the milk replacer, reconstituted at the rate of one part to ten parts of warm water and fed in open pails. No hay or grain was fed. Water and iodized salt were offered ad libitum. The calves were housed in a heated barn in elevated box-type pens with metal-grid floors. In pens such as these, the calves could move about freely and the metal-grid afforded satisfactory footing. Previous attempts to confine calves in stanchions had proved to be unsatisfactory.

Between the ages of 2 and 3 wk., digestibility trials of at least six days' duration were initiated. Fecal collections were made by attaching polyethylene bags to the calves in the manner described by Noller *et al.* (15). In the event of diarrhea during the preliminary period of the digestion trial, the trial was postponed. If diarrhea occurred during the digestion trial the data were, with one exception, discarded. The exception was made in Period Two of the tallow treatment in which the feces were collected for three days, discarded for two days because of diarrhea, and again collected for three days. During the preliminary period of the digestion trials, a carmine marker was fed with the night feeding. The time from the feeding of the marker until its initial appearance in the feces was recorded as transit time.

Feeds were analyzed for fat by extraction in a Soxhlet apparatus. Aliquots of the fat homogenates were analyzed by adding subsamples to tared extraction flasks filled with shredded asbestos. The flasks were dried, and the unevaporated residue of the subsample was calculated as total fat. Samples of dried feces were extracted for 48 hr. with ethyl ether in a Soxhlet apparatus. Following this preliminary extraction, the sample was ground and analyzed for total fat including soaps, by an adaptation of the Saxon method (18). The sum of the extractions was used in calculating the digestion coefficients.

Statistical analyses were made of the digestibility data as follows: An arcsin transformation was made of the digestion coefficients to correct for heterogenous variance as well as non-normality. Missing plots were estimated and an analysis of variance was made by the methods outlined by Snedecor. The upward bias of the treatment mean square caused by the calculation of missing plots was corrected (19). If a significant F value was obtained, the multiple range test of Duncan (6) was used to test the significance of individual means. Except for the transformation, the transit time data were analyzed in a similar manner.

## RESULTS AND DISCUSSION

The apparent digestion coefficients of total fat of the experimental diets are given in Table 2. As has been mentioned, the butter treatment was not assigned in the first block. The other missing observations resulted from removal of the animals from experiment, due to scours. Statistical analysis of Experiment I showed the fat digestibility to be significantly higher at the 1% level for the animals fed the diets containing homogenized coconut fat plus crude soybean lecithin and coconut fat plus crude soybean lecithin, than for the animals fed any of the other experimental diets. Tallow plus lecithin was better digested than tallow. In Experiment II (Table 2), the calves fed grease plus lecithin digested the fat significantly better (1% level) than those fed grease alone. No other statistically significant difference was noted.

Apparently, when skimmilk is supplemented with an unhomogenized fat without an emulsifying agent, the fat is rather poorly utilized. However, the inclusion of lecithin in a high-fat diet appears to increase the digestibility of the fat. Because of the excellent utilization of coconut fat plus lecithin in this experiment, homogenization could not be expected to improve its utilization by any appreciable amount. Rather inexact data were collected in the one digestion trial in which the basal mixture was supplemented with homogenized tallow plus lecithin. Due to a sampling problem it was calculated from chemical analyses that between 370

TABLE 2

Apparent digestibility of fat and transit time of diets by dairy calves

| Diet | Block | | | | | Av. | Transit time |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Experiment I | | | | | | | *(hr.)* |
| 1. Tallow | 64.6 | 52.4 | | 53.8 | 66.2 | 59.2 | 22 |
| 2. Coconut fat | 66.0 | 60.1 | | 64.4 | 71.3 | 65.4 | 25 |
| 3. Butter | | 73.3 | 52.8 | 66.1 | 76.9 | 67.3 | 22 |
| 4. Tallow + lecithin | 85.0 | 68.9 | 57.1 | 77.5 | | 72.1 | 17 |
| 5. Coconut fat + lecithin | 96.0 | 97.9 | 90.4 | 98.2 | 95.4 | 95.6 | 21 |
| 6. Coconut fat + lecithin (homogenized) | 90.4 | 96.1 | 96.9 | 96.0 | | 94.9 | 22 |
| Experiment II | | | | | | | |
| 7. Choice white grease | 59.1 | 44.2 | 51.1 | | | 51.5 | |
| 8. Choice white grease + lecithin | 77.8 | 81.2 | 79.2 | | | 79.4 | |

and 550 g. of fat were fed. However, since only 25 g. of fecal fat were collected, the digestibility of the tallow homogenate must have been between 93 and 96%. This single observation is indicative that homogenization does indeed improve fat utilization of a poorly digested fat and helps explain the good growth responses that have been obtained when homogenized tallow is fed to calves.

The low digestion coefficients obtained from butter are of interest in view of the high digestion coefficients that have been reported for the ether extract of liquid and of spray-dried whole milk. These observations further suggest that the physical form of fat is important for maximum utilization by the calf. It should be noted, however, that significant amounts of the lecithins of whole milk are not present either in churned butter or in skimmilk, but are concentrated in the buttermilk.

It is apparent that crude soybean lecithin improves the digestion of fats by young dairy calves. While this improvement would seem to be due to the action of the lecithin as an emulsifying agent, it may result from some other action or a constituent of the crude lecithin. It is of interest to note that the digestion of the coconut fat, a low-melting fat, was improved more than was the digestion of tallow, a high-melting fat. Auger *et al.* (1) observed that the digestibility of high-melting fats by rats was improved more than that of low-melting fats by the inclusion of lecithin in the diet. However, these workers fed fats with higher melting points than we used, and they observed sizable differences in digestibility between the different fats fed without lecithin.

The transit times of the diet that were obtained with the carmine marker are presented in Table 2. Transit times of the rations were found to vary widely from calf to calf. Analysis of variance of the data gave a nonsignificant F of 1.3. Thus, the speed of passage did not seem to be an important factor in fat digestion in this experiment.

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# CHANGES IN THYROID $I^{131}$ RELEASE RATE DURING GROWTH IN HOLSTEIN, BROWN SWISS, AND JERSEY CALVES AT CONSTANT ENVIRONMENTAL TEMPERATURES OF 50 AND 80° F.[1, 2]

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## SUMMARY

Studies on the effect of age and temperature on thyroid $I^{131}$ release rate on growing calves were conducted under the controlled environmental conditions of the Missouri Climatic Laboratory.

At both temperatures and for all calves, thyroid $I^{131}$ activity increased with age, whereas thyroid $I^{131}$ activity per unit weight decreased with age. Although all calves showed the same trends, breed differences in the latter relationship were highly significant, Jerseys having the highest ratios, followed by Brown Swiss and Holsteins. The thyroid $I^{131}$ release rate of Jersey calves was approximately twice as great as the rate for Holstein and Brown Swiss, throughout this growth study; however, only the breed-age interaction was significant.

Thyroid activity per unit weight was higher at 80° F. than at 50° F., with highly significant breed differences occurring, and varied significantly as a function of the interaction between temperature and age. Thyroid $I^{131}$ activity per unit surface area showed no effect of temperature alone, but did increase slightly with increments in both age and temperature. Breed differences were significant in the ratio of thyroid $I^{131}$ activity to surface area.

The exponents ($b$ values) of the parabolic equation expressing the relationship of thyroid $I^{131}$ activity to body weight proved to be significantly higher at 80 than at 50° F. There were significant breed differences in percentage increase in thyroid $I^{131}$ activity per 1% increase in body weight, the Jerseys' rate being higher than those of the Brown Swiss or Holsteins.

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Thyroid $I^{131}$ activity, believed to be an important factor in growth and milk production, was measured on growing dairy calves in the Missouri Climatic Laboratory. The objective was to determine the effects of 50 and 80° F. constant environmental temperature on the changes in thyroid activity during growth (1–12 mo.).

The studies of Johnson *et al.* (8, 9) on rabbits showed that relatively high constant environmental temperatures depressed thyroid $I^{131}$ release rate. Blincoe (1) demonstrated a similar effect upon beef cattle and Brown-Grant (3) provided evidence that cold environmental temperatures increased the thyroid $I^{131}$ release rate of rats.

## EXPERIMENTAL CONDITIONS AND METHODS

*Experimental conditions.* These studies were conducted under the controlled environmental conditions of the Missouri Climatic Laboratory. The laboratory consists of two independently controlled chambers; one chamber was maintained

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at a constant temperature of 50° F. and the other at a constant temperature of 80° F. Humidity, air velocity, and illumination remained constant.

Holstein, Brown Swiss, and Jersey heifer calves were used in these studies. Three calves of each breed were placed in pens in each chamber at about 2 wk. to 1 mo. of age and were maintained there until the end of the experiment (about 12 mo. of age). Feed composition and management were the same for each chamber.

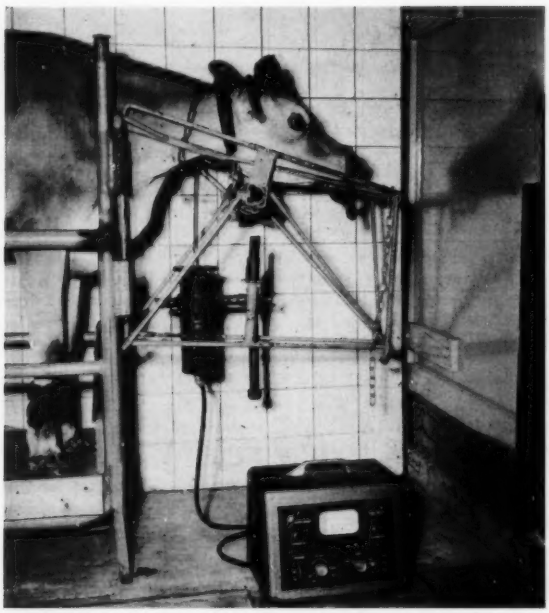

FIG. 1. Thyroid $I^{131}$ activity counting apparatus. The stall is portable; the wheels are removed during the counting procedure.

Calves were fed alfalfa hay ad libitum and grain according to a program similar to that suggested by Herman (5).

*Experimental procedures.* Approximately every 6 wk. the calves were injected intravenously with carrier-free Na $I^{131}$. The dose level was 100 $\mu$c. until the calves were 6 mo. old, then 200 $\mu$c. throughout the remainder of the experiment.

Figure 1 indicates the method for determination of the thyroid $I^{131}$ release rate. The restraining stall and head-holder is portable and the wheels are removed during the counting procedures. The counting stall was moved into the temperature chambers and counts were made daily on the animals, a procedure to which the animals readily became accustomed. An NRD scintillation detector (1 in. crystal) with a 30-cm. spacer was used for the counting procedures. Maximum counts per minute were recorded by a Nuclear Chicago rate meter. With this counting procedure the standard activity was determined (a known amount,

actually one-half of the dose given the animal) at a distance approximately equal to that of the thyroid from the detector. Counts as indicated were made of the thyroid region and, after correction for the standard dilution factor and room background level, the data were expressed as per cent of standard dose. Counts were made at the statistical error level of 0.01. Duplicate readings were taken and the values averaged.

The values were plotted on semilogarithmic paper and the slopes determined from daily data taken from three to approximately 30–35 days after injection of Na $I^{131}$.[3] The slopes or loss of activity in the thyroid region were determined by the method of least squares to express the rate of thyroid release of $I^{131}$ activity. This slope, or $K$, is equal to $K_4$ as reported by Blincoe and Brody (2).

In addition to thyroid $I^{131}$ activity, many physical and physiological measurements were made on the growing dairy calves. These measurements included: body weight—twice weekly; heart girth and wither height—bimonthly; surface area (surface integrator method), hair density (beta gauge), hair length and color—every 6 wk.; rectal temperature, respiration, and pulse rate, feed and water consumption—daily; heat production, respiratory, surface (Capsule), and total vaporization—weekly; and red-cell volume ($Cr^{51}$), butanol soluble $I^{131}$, and blood glutathione (GSH)—every 6 wk.

## DATA AND DISCUSSION

*Changes in thyroid $I^{131}$ activity versus age.* The thyroid $I^{131}$ release rate of the three breeds differed, as shown in Figure 2 and in Table 1.

TABLE 1

Analysis of variance of thyroid $I^{131}$ activity

| Source of variation | Df | Mean square | F | P |
|---|---|---|---|---|
| Temperature | 1 | 8.0716 | .3750 | n.s. |
| Age | 6 | 36.8352 | 43.5281 | s(.01) |
| Breed | 2 | 75.4008 | 3.5029 | n.s. |
| Temperature × age | 6 | 3.7130 | 4.3876 | s(.01) |
| Temperature × breed | 2 | 13.2191 | .6141 | n.s. |
| Age × breed | 12 | 3.0831 | 3.6433 | s(.01) |
| Temperature × age × breed | 12 | 0.5141 | .6075 | n.s. |
| Animal in TB | 12 | 21.5250 | 25.4361 | s |
| Age × animal in TB | 72 | 0.8462 | ..... | ..... |

An analysis of variance, performed according to the outline recommended by Schultz (11), demonstrated that the effect of age upon thyroid $I^{131}$ activity was highly significant. Breed and temperature differences in thyroid $I^{131}$ activity per se were not significant. Although the interaction between temperature and breed was insignificant as well, the interactions between age and temperature and between age and breed were significant.

Thyroid $I^{131}$ activity increased more with age at 80 than at 50° F.; nevertheless, the early data points probably influenced this trend. The early data

[3] Reutilization correction factors were not used, since accurate values for calves of various ages and environmental temperature conditions were not available.

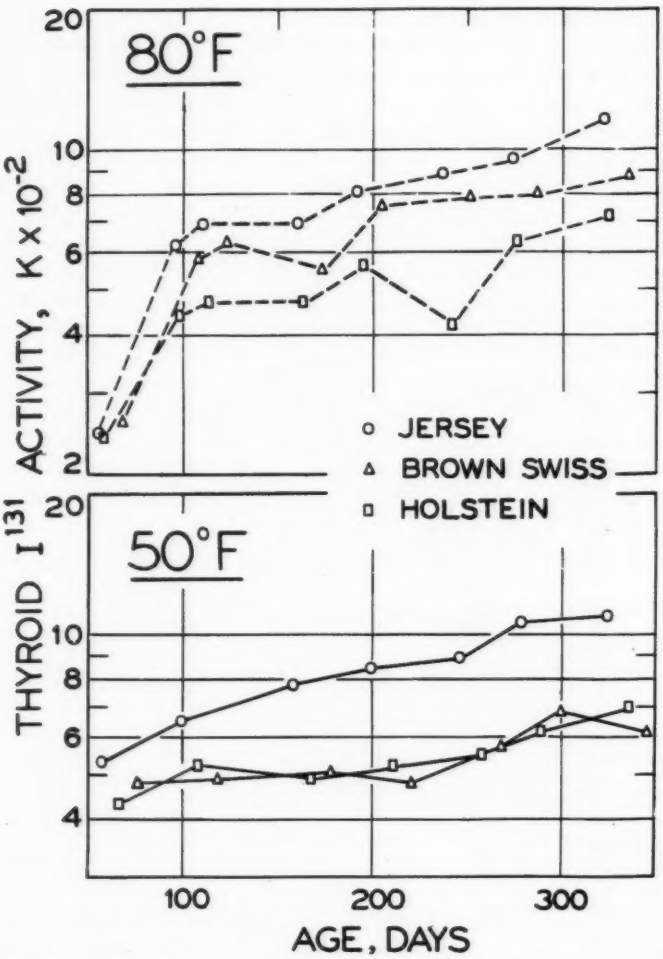


FIG. 2. Thyroid $I^{131}$ activity of Jersey, Brown Swiss, and Holstein calves during growth at 50 and 80° F.

points for the calves in the 50° F. room may be high, because of the fact that the animals were experiencing a considerable constant cold exposure for their relatively small body size. The initially high thyroid $I^{131}$ activity may be a reflection of their extra heat requirements due to greater surface area per unit weight. It is also probable that the animals in the 80° F. chamber were more heat-stressed during the early growth period and, consequently, depressed in their thyroid activity. However, observations of short-period exposures, made by Johnson (7) at the conclusion of the growth experiment, indicated significant 80–50° F. differences. The present similarity in thyroid $I^{131}$ activity is believed due to

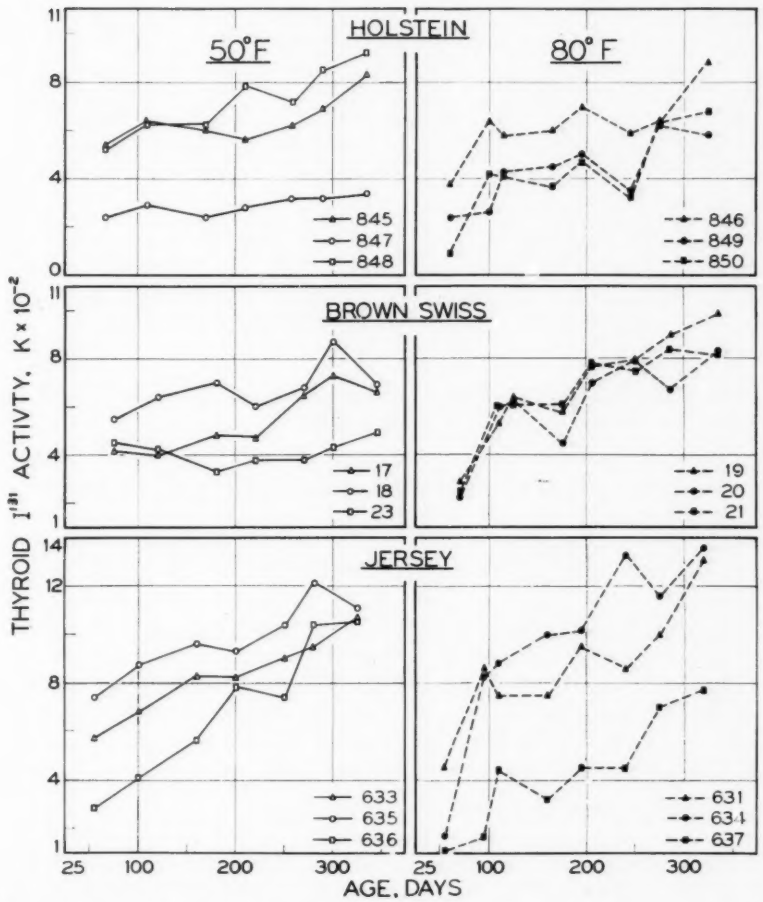


FIG. 3. Thyroid $I^{131}$ activity for individual Holstein, Brown Swiss, and Jersey calves during growth at 50 and 80° F. Graphs indicate considerable individual differences in some breeds, which were constant throughout the growth study.

the relatively much longer period of acclimation, or adaptation of the growing animal to the environment. The exact mechanisms of acclimation, or adaptation, in cattle are unknown at the present time.

Hoesslin (6) observed a similar adaptation phenomenon from results of studies on two-litter mate dogs—one raised at 32° C. and the other at 5° C. Even though there was an enormous temperature difference between the body and the air, the 5° C.–reared dog's metabolism was only 12% higher. However, the cold-reared dog's fur was three times heavier than that of the 32° C. animal. The dairy calves in this experiment appeared to have compensated by this as well

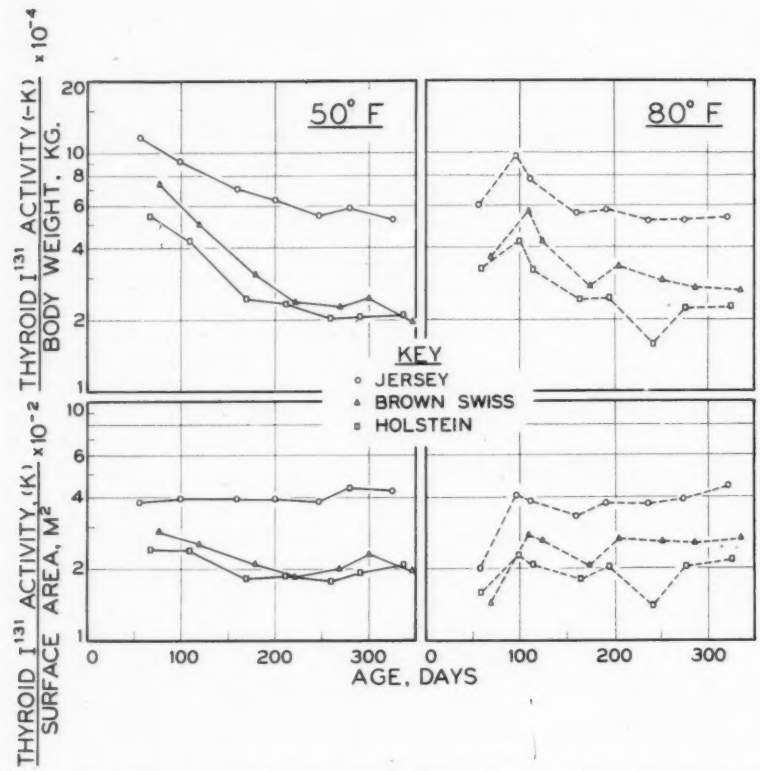


FIG. 4. Thyroid $I^{131}$ activity per unit surface area and per unit body weight for Jersey, Brown Swiss, and Holstein calves raised at 50 and 80° F.

as by other physical and physiological factors as indicated by unpublished hair density data on these calves. For example, the average hair densities for the 50 and 80° F. dairy calves were 12.3 mg/cm and 9.2 mg/cm, respectively.

The influence of the hair coat on mammals is further emphasized by the work of Freinkel and Lewis (4), in which the thyroid activity was reported higher in shorn sheep than in unshorn sheep at the same cold environmental temperature.

*Individual animal differences in thyroid $I^{131}$ activity.* Striking individual differences were apparent in the 50° F. Holsteins and the 80° F. Jerseys, as indicated graphically in Figure 3. Note the lower level of thyroid activity of Holstein 847 and Jersey 637. These within-breed differences were consistent throughout the growth study. The relationship of these individual differences to heat tolerance or level of production (growth) will be reported later.

*Thyroid activity per unit weight.* In the ratio of thyroid activity to unit weight (upper section of Figure 4 and Table 2), the Jerseys again had the highest values, the Brown Swiss, intermediate, and the Holsteins, the lowest values per

unit weight throughout the study of growth. These breed differences were statistically significant at the 0.01 level of probability.

TABLE 2

Analysis of thyroid $I^{131}$ activity per unit weight

| Source of variation | Df | Mean square | F | P |
|---|---|---|---|---|
| Temperature | 1 | 8.8956 | 1.0193 | n.s. |
| Age | 6 | 33.4009 | 29.2117 | s(.01) |
| Breed | 2 | 181.4478 | 20.7915 | s(.01) |
| Temperature × age | 6 | 10.0190 | 8.7624 | s(.01) |
| Temperature × breed | 2 | 2.9921 | .3429 | n.s. |
| Age × breeds | 12 | .7986 | .6984 | n.s. |
| Temp. × age × breeds | 12 | .6916 | .6049 | n.s. |
| Animal in TB | 12 | 8.7270 | 7.6325 | s |
| Age × animal in TB | 72 | 1.1434 | ..... | ..... |

In younger calves (1–3 mo.), it is generally known that all metabolic functions are the highest per unit weight. At this age the thyroid $I^{131}$ activity is approximately twofold greater than for animals approximately 1 yr. old. This definite trend for thyroid activity per unit weight to decrease with increasing age is evident at both the 50 and 80° F. temperatures. The difference in this ratio as a function of temperature was not significant, whereas the difference as a function of the interaction of age and temperature was again highly significant, with a probability of 0.01.

*Relationship of thyroid activity to surface area ($M^2$).* A constant relationship of thyroid activity to growth is shown in the lower section of Figure 4. This graph relates a productive or compensatory productive mechanism such as thyroid activity to the major heat conservation or dissipating surface of the animal. Again, as presented in Table 3, the ratios for the three breeds differ much in the same order, and they are significantly different at the 0.01 level of probability.

TABLE 3

Analysis of thyroid $I^{131}$ activity per unit surface area

| Source of variation | Df | Mean square | F | P |
|---|---|---|---|---|
| Temperature | 1 | 20.9649 | .0462 | n.s. |
| Age | 6 | 47.6608 | 1.0478 | n.s. |
| Breed | 2 | 4551.0574 | 10.0310 | s(.01) |
| Temperature × age | 6 | 234.6041 | 6.9295 | s(.01) |
| Temperature × breed | 2 | 152.1525 | .3354 | n.s. |
| Age × breed | 12 | 9.1173 | .2693 | n.s. |
| Temperature × age × breed | 12 | 33.2174 | .9811 | n.s. |
| Animal in TB | 12 | 453.7005 | 13.4010 | s |
| Age × animal in TB | 72 | 33.8558 | ..... | ..... |

Figure 5 and surface area data, Kennedy *et al.* (10), offer some explanation for this relationship. Kennedy demonstrated that the logarithmic relationship of thyroid activity to body weight which varies with the breed and temperature is similar to the logarithmic relationship of surface area to body weight. Although the ratio of thyroid $I^{131}$ activity per surface area does not vary appreciably

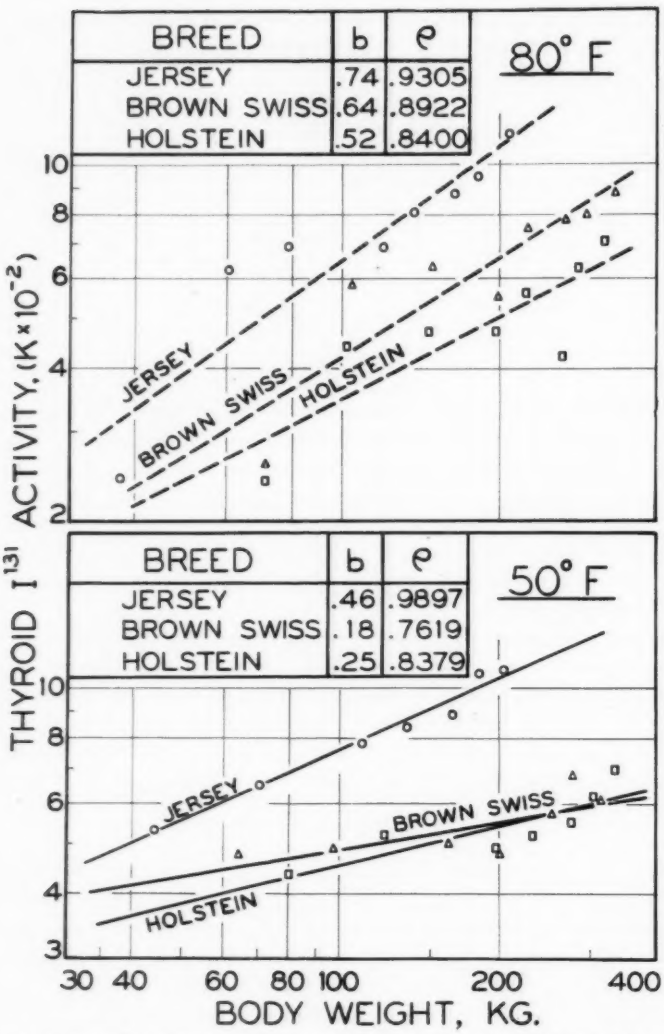


FIG. 5. Thyroid $I^{131}$ activity versus body weight. Levels of significance of the correlation coefficients $r$ were as follows: 50° F.—Jersey 0.01, Brown Swiss 0.05, and Holstein 0.05; 80° F.—Jersey 0.01, Brown Swiss 0.05, and Holstein 0.01.

either with age or with temperature, it does vary significantly, at the 0.01 level, as a function of the interaction between age and temperature.

In interpreting these ratios (thyroid $I^{131}$ activity per unit weight and per unit surface area), the magnitude of the denominators must be considered. At both temperatures, the body weights of the Jerseys were considerably lower than

those of the Holsteins and Brown Swiss; the surface area values of the Jerseys also were considerably lower. The body weights and surface area values of the Brown Swiss and Holsteins were fairly similar to each other.

Considerable evidence indicates that thyroid activity is determined by the tissue demand for the thyroid hormone (12). Since the thyroid function, like metabolism, represents a generalized activity, it appears to be justifiable to express the data in the given ratios.

*The logarithmic relationship of thyroid $I^{131}$ activity to body weight.* The regression lines in Figure 5, obtained from averages of three individuals in each breed at each temperature, are expressed by the equation $Y = aX^b$, with the thyroid activity as the dependent variable. An increase in weight $(X)$ by 100% is associated with an approximate differential increase in $Y$ (thyroid activity) by as low as 0.18 to as high as 0.74, respectively, for the 50° F. Brown Swiss and 80° F. Jerseys. Mean exponents for the 80° F. Jerseys were: 0.74 as compared to 0.46 for the 50° F. Jerseys; Brown Swiss, 0.64, at 80° F. as compared to 0.18 at 50° F.; and 0.52 for Holsteins at 80° F. and 0.25 for the 50° F. group.

Table 4 shows a statistical comparison of the individual $b$ values.

The relationship of thyroid activity to body weight was significantly different (.05 level of probability) at the two temperatures. Breed differences also were highly significant (P = .01).

TABLE 4

Analysis of variance of "b" values from thyroid $I^{131}$ activity vs. body weight (kg.)

| Source of variation | Df | Mean square | F | P |
|---|---|---|---|---|
| Temperature | 1 | .00032496 | 5.9978 | s(.05) |
| Breed | 2 | .00134168 | 24.7634 | s(.01) |
| Temperature × breed | 2 | .00001645 | .3036 | n.s. |
| Animal in TB | 12 | .00005418 | ..... | ..... |

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# OBSERVATIONS ON THE NUTRITION OF *BACTEROIDES SUCCINOGENES*—A RUMINAL CELLULOLYTIC BACTERIUM

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**SUMMARY**

Studies on five strains of *Bacteroides succinogenes*, including one recent isolate, indicated that biotin was essential for growth of four strains and highly stimulatory for the other. *p*-Aminobenzoic acid stimulated the growth of two strains. No other vitamins had an effect on growth. Ammonia was essential for growth even in the presence of 19 amino acids, a mixture of purines and pyrimidines, and all B vitamins. None of the amino acids were essential for growth, and good, but somewhat delayed, growth was obtained when cysteine or glutathione was the only source of amino acids available. Studies on Strain S85 indicated that cysteine or sulfide could serve as the sole source of sulfur. The sulfur requirement was not adequately studied, as the only reducing agents found that allowed good growth in chemically defined media were those that also served as a sulfur source. The essentiality of $PO_4^{---}$, $NH_4^+$, $Mg^{++}$, $Ca^{++}$, $K^+$, and $Na^+$ was demonstrated and the levels required were established for Strain S85. Although glucose was used as an energy source in most experimental media, it was demonstrated that good growth and cellulose digestion occurred in a medium containing only cellulose, *p*-aminobenzoic acid, biotin, cysteine, alanine, phenylalanine, valerate, isobutyrate, a carbonic acid–bicarbonate buffer, resazurin, and minerals. The results indicate the relatively great ability of *B. succinogenes* to synthesize cellular constituents and, also, how well this bacterium is adapted to the environment of the rumen.

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It is now generally known that the nutrient requirements of ruminants for efficient utilization of feeds are intimately associated with reactions occurring in the rumen as well as the essential and stimulatory nutrients of ruminal microorganisms. Also, knowledge of the nutrients involved in growth is essential to an understanding of the ecology of an organism. While animal feeding trials and in vitro experiments involving the mixed microbial population have been used to good advantage in advancing knowledge of the nutrition of ruminal organisms, it is evident that studies of pure cultures of judiciously selected species may bring forth information difficult to obtain or difficult to interpret with other methods.

Data of Hungate (20) and others (7, 8, 9) have indicated that *Bacteroides succinogenes* is one of the more important cellulolytic species in the rumen. Previous studies on the nutrient requirements for growth of this organism indicated that anaerobic conditions (9, 20), carbon dioxide, or bicarbonate (9), an energy source such as glucose, cellobiose, or cellulose (9, 20), and a two-component volatile fatty acid mixture (10) are required for growth.

The present work was initiated to obtain knowledge of the B vitamin, nitrogen, sulfur, and mineral requirements of this species.

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## METHODS

The strains studied were S61, S85, S121, and M13, many characteristics of which were reported previously (9, 10), and a more recent isolate, CB40, that was isolated in April, 1957, from a steer on a feed-lot, bloat-provoking ration. The latter strain was only presumptively identified as *B. succinogenes* on the basis of morphology, nonmotility, gram reaction, cultural characteristics, relations to oxygen, digestion of cellulose, final pH in glucose medium, and nonproduction of gas and $H_2S$.

The anaerobic methods used to prepare the media and grow the bacteria were essentially the same as described by Bryant and Doetsch (10), except as indicated below. The composition of the inoculum medium is shown in Table 1.

TABLE 1
Composition of inoculum medium[a]

| | *(mg/100 ml.)* | | *(mg/100 ml.)* |
|---|---|---|---|
| $KH_2PO_4$ | 90 | Glucose | 300 |
| NaCl | 90 | Cysteine · HCl · $H_2O$ | 100 |
| $(NH_4)_2SO_4$ | 90 | Casein hydrolysate[b] | 200 |
| $CaCl_2$ | 9 | Thiamin · HCl | 0.2 |
| $MgSO_4$ | 9 | Pyridoxamine · HCl | 0.1 |
| $FeSO_4 \cdot 7\ H_2O$ | 4 | Ca-pantothenate | 0.2 |
| $MnSO_4 \cdot H_2O$ | 0.15 | Riboflavin | 0.2 |
| $CoCl_2 \cdot 6\ H_2O$ | 0.15 | Nicotinamide | 0.2 |
| $Na_2CO_3$ | 400 | *p*-Aminobenzoic Acid | 0.01 |
| Sodium *n*-valerate | 7.44 | Biotin | 0.005 |
| Sodium isobutyrate | 3.33 | Folic Acid | 0.005 |
| Resazurin | 0.1 | Cobalamin | 0.0005 |

[a] The medium was in equilibrium with $CO_2$ gas that was passed through a column of hot reduced copper filings to remove traces of $O_2$.
[b] Nutritional Biochemicals Corporation vitamin-free casein hydrolysate (enzymatic).

The strains were carried with daily transfers of one 4-mm. loop in 5 ml. of inoculum medium 1 in 13 by 100-mm. pyrex culture tubes. At least seven transfers were made before inocula were prepared. They were inoculated at approximately 4 P.M., incubated at 37° C. until 8 A.M., and left at room temperature until inoculum was prepared. Leaving them at 37° C. for longer periods resulted in lysis of cells (10) and erratic growth. Inoculum was prepared by centrifuging down the cells, decanting the medium, and making up the cells to an optical density (O.D.) of 0.1 with anaerobic dilution solution containing 1.2% $KH_2PO_4$, 1.2% NaCl, 0.1% cysteine · HCl, and 0.4% $Na_2CO_3$, and equilibrated with $CO_2$ gas. Sterile solutions of cysteine · HCl and $Na_2CO_3$ were prepared and added to the solution after autoclaving, as previously described (10). O.D. was determined in calibrated 13- by 100-mm. pyrex culture tubes using a Spectronic 20 colorimeter set at 600 m$\mu$. Except when centrifuging, the cells were maintained under $CO_2$ in rubber-stoppered tubes. One-tenth milliliter was used to inoculate each 5 ml. of medium. Preliminary experiments indicated Strain S85 required biotin. Using biotin-deficient medium, it was found that washing the cells two or three times resulted in no less carry-over of biotin.

The basal medium for the study of vitamin requirements was the same as the inoculum medium, except that vitamins were deleted. The medium was tubed in

4-ml. amounts, so that addition of 1 ml. of the various vitamin solutions brought it to final concentration. Vitamin mixtures were sterilized for 10 min. at 15 lb. in absorbent cotton-plugged tubes or in tubes with aluminum caps. On removal from the autoclave, the tubes were gassed well with $CO_2$ and closed with sterile rubber stoppers to prevent entrance of $O_2$ until ready for addition of the vitamin solutions to the basal medium.

The basal medium for determining nitrogen requirements was similar to the inoculum medium except that L-amino acids were substituted for the casein hydrolysate. Amino acids included were serine, alanine, tryptophane, aspartic acid, arginine · HCl, histidine · HCl · $H_2O$, lysine · HCl, valine, glycine, proline, hydroxyproline, leucine, isoleucine, threonine, methionine, and phenylalanine at concentrations of 0.01%, and glutamic acid and tyrosine at 0.02% and 0.0017%, respectively. Amino acids were added to the medium before autoclaving. Specific variations in media are given with the results.

In studying the mineral requirements of Strain S85, a number of changes were made in the experimental procedure. Cells used as inoculum were grown in the same manner as above, except that a clarified rumen fluid (CRF) glucose medium was used. This medium was of the following composition: 20% CRF, 0.0005% indigo carmine, and 0.09% $(NH_4)_2SO_4$. Cysteine, glucose, carbon dioxide, and sodium carbonate were added as in the original inoculum medium (Table 1). The CRF was prepared from freshly collected rumen fluid from a cow on an alfalfa hay–grain ration. It was filtered through gauze, allowed to stand until particles rose to the surface or sedimented and the clearer liquid was siphoned off. This was centrifuged at approximately 14,000 r.p.m. for 20 min. and the clear supernatant was sterilized under $CO_2$ by autoclave (15 lb/20 min.) and refrigerated until used.

The basal medium in which minerals were varied contained glucose, indigo carmine, $Na_2CO_3$, and cysteine as in the CRF medium. Other ingredients were as follows in milligrams per 100 ml.: sodium isovalerate, 3.72: sodium valerate, 7.44; *p*-aminobenzoic acid, 0.01; biotin, 0.005; and alanine, isoleucine, serine, phenylalanine, tryptophane, glutamic acid, lysine, arginine, and proline, ten of each. The media were adjusted to pH 6.5 with NaOH, $H_2SO_4$, or HCl before autoclaving (10 min/15 lb.) and adding $Na_2CO_3$ and cysteine. After the pH was adjusted, it was held under a gas mixture of 80% $CO_2$ and 20% $H_2$. It was tubed in 4-ml. amounts, so that addition of 1 ml. of mineral solution brought the ingredients to final concentration.

Minerals used were of A.C.S. quality and each salt was prepared individually in screw-cap bottles and autoclaved at 15 lb. for 10 min. For preparation of the various mixtures, the appropriate amounts of the individual solutions were mixed in sterile aluminum-capped tubes. They were steamed in an Arnold sterilizer for 10 min. or longer and gassed with the $CO_2$-$H_2$ mixture to remove $O_2$. One-milliliter additions were made to the basal medium.

Water used to double-rinse glassware and in culture media was distilled, passed through a column of Amberlite MB-3 ion exchange resins, distilled from Pyrex glass, and stored in polyethylene bottles.

The washed inoculum was prepared as follows: About 0.8 ml. of 18-24 hr. culture was diluted to 3 ml. with the basal medium, with water added in place of the mineral mixture. The cells were centrifuged down at about 12,000 r.p.m. The pellet of cells was washed with the medium and then resuspended and centrifuged down again. The supernatant was decanted and the cells were resuspended and diluted out to O.D. 0.1. Each 5 ml. of medium was inoculated with 0.1 ml. of the suspension.

The O.D. of growth media with indigo carmine added in place of resazurin was determined, utilizing a wave length of 475 m$\mu$.

## RESULTS

*Vitamin requirements.* Single deletion of vitamins from the medium indicated that only biotin and *p*-aminobenzoic acid (PABA) had an effect on growth. Some of the results are shown in Table 2. Biotin was essential for growth of four strains and quite stimulatory for Strain S121. That biotin was not carried over in the inoculum with Strain S121 was indicated by the fact that growth occurred through five successive daily serial transfers in this medium. PABA was stimulatory to Strains S61 and CB40. It was much more stimulatory when added to the medium containing biotin only than when all of the other vitamins were present. Strains S85, M13, and S121 exhibited maximal growth when biotin was the only vitamin present and Strains S61 and CB40 grew well with only biotin and PABA present.

TABLE 2

Effect of vitamins on growth of strains of *B. succinogenes*

| Vitamins added | Growth (O.D. × 100) | | | | |
|---|---|---|---|---|---|
| | Strain S85 | Strain M13 | Strain S121 | Strain S61 | Strain CB40 |
| All vitamins[a] | 96 (24)[b] | 105 (24) | 82 (24) | 96 (24) | 92 (27) |
| All except biotin | 1 (24) | 1 (24) | 28 (48) | 3 (24) | 3 (24) |
| All except PABA | 93 (24) | 102 (24) | 79 (24) | 52 (48) | 44 (112) |
| Biotin and PABA | 92 (24) | 100 (24) | 82 (24) | 96 (27) | 82 (27) |
| Biotin | 96 (24) | 105 (24) | 80 (24) | 17 (27) | 7 (24) |

[a] Thiamine · HCl, pyridoxamine · HCl, Ca-pantothenate, riboflavin, nicotinamide, PABA, biotin, folic acid, and cobalamin.

[b] The numbers in parentheses refer to the hours of incubation for the maximum O.D. reading.

TABLE 3

Effect of certain vitamins on growth of Strain S121[a]

| Vitamins added | O.D. × 100 | Vitamins added | O.D. × 100 |
|---|---|---|---|
| None | 2 (16) | Nicotinamide | 57 (116) |
| All except biotin | 62 (88) | Cobalamin | 54 (88) |
| Biotin | 92 (24) | Thiamine | 49 (116) |
| PABA | 47 (116) | Pyridoxamine | 60 (96) |
| Riboflavin | 48 (116) | Folic Acid | 42 (88) |
| Ca-pantothenate | 48 (88) | | |

[a] See footnotes of Table 2.

Data in Table 3 show that no appreciable growth of Strain S121 occurred when all vitamins were deleted. Yet slow and limited growth occurred with each vitamin added singly to the medium, except in the case of biotin, which allowed optimum growth. These observations might suggest that each of the vitamins either partially replaces biotin or that the medium was contaminated with biotin. However, it does not seem logical that all of these vitamins might partially replace biotin. The fact that the control medium with no vitamins added did not allow appreciable growth makes it seem evident that if biotin was a contaminant its source was the other individual vitamins. It likewise does not seem possible that this could occur.

Figure 1 shows the response of Strain S85 to different levels of biotin.

*Nitrogen requirements.* In preliminary experiments it was shown that purines and pyrimidines previously included in the medium (10) had no appreciable effect on growth. Data in Table 4 indicate that 18 L-amino acids can replace the

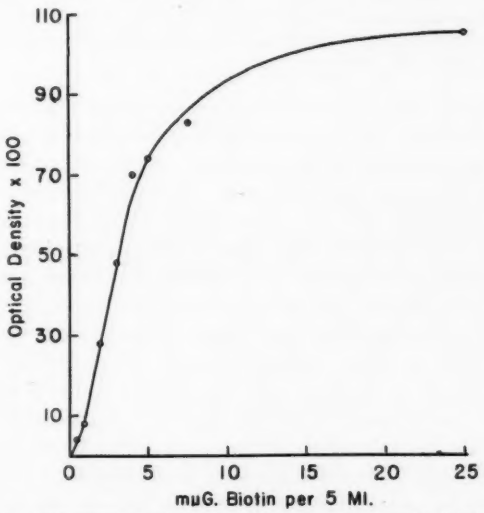


FIG. 1. The response of Strain S85 to varying levels of biotin.

casein hydrolysate in the growth of all five strains and that good, but somewhat delayed, growth is obtained when cysteine or glutathione, added as reducing agent, is the only source of amino acids in the medium. Experiments outlined below indicate that good growth can be obtained with sulfide used in place of cysteine. Thus, no amino acid is essential for growth.

The results indicate that the number of vitamins essential for growth is not increased when all amino acids except cysteine are deleted from the medium. In Experiment 2 of Table 4, PABA and biotin were the only vitamins present.

Data in Experiment 1 of Table 4 indicate that growth did not occur when $Na_2SO_4$ replaced $(NH_4)_2SO_4$ in the medium. Other experiments showed that

TABLE 4

Effect of some nitrogen sources on growth of strains of *B. succinogenes*[a]

| Ingredients added to basal medium | Growth (O. D. × 100) Strain S85 | Strain M13 | Strain S121 | Strain S61 | Strain CB40 |
|---|---|---|---|---|---|
| Experiment 1[b] | | | | | |
| Casein hydrolysate, $(NH_4)_2SO_4$ | 100(24) | 100(24) | 96(24) | 96(40) | 90(40) |
| 18 L-amino acids, $(NH_4)_2SO_4$ | 82(24) | 96(24) | 96(40) | 88(40) | 92(40) |
| 18 L-amino acids, $Na_2SO_4$ | 4(51) | 5(24) | 3(51) | 2(95) | 3(68) |
| $(NH_4)_2SO_4$ | 77(68) | 77(51) | 80(68) | 80(76) | 1(68) |
| $Na_2SO_4$ | 0(119) | 0(119) | 0(119) | 0(119) | 0(119) |
| Experiment 2[c] | | | | | |
| Casein hydrolyysate + cysteine | 110(25) | 105(18) | 89(18) | 96(40) | 96(25) |
| Cysteine | 100(42) | 89(50) | 82(50) | 105(40) | 40(108) |
| Glutathione (0.1%) | 100(42) | 82(42) | 82(42) | 96(40) | 60(42) |

[a] See Footnote [b], Table 2.

[b] The basal medium was that shown in Table 1, except that casein hydrolysate and $(NH_4)_2SO_4$ were deleted and 0.5 mg. each of adenine, guanine, uracil, xanthine, and thymine per 100 ml. of medium were added.

[c] The basal medium was that shown in Table 1, but minus casein hydrolysate, cysteine, and all vitamins except PABA and biotin.

the $Na_2SO_4$ was not toxic when included in media in addition to $(NH_4)_2SO_4$. These results indicate that ammonia is essential for growth even in the presence of 19 amino acids, purines, and pyrimidines, and all of the B vitamins.

Using Strain S85, attempts were made to determine the effects of various amino acid mixtures on growth in the medium in Table 1 modified by deletion of casein hydrolysate. Cysteine was always present as reducing agent. Growth was good regardless of the amino acids added, but often a long lag occurred before growth was initiated. In early work it was found that alanine was the only deletion that slowed growth when single deletions were made from mixtures of all 18 amino acids or from a mixture of ten. When various mixtures of five or less amino acids were present, it was found that alanine alone did not support rapid growth, but rapid growth was obtained when alanine plus phenylalanine and/or tryptophane were present. Further experiments indicated that growth was quite slow if both phenylalanine and tryptophane were deleted from the complete mixture, or from mixtures containing ten or less amino acids. Tyrosine would not replace the two other aromatic amino acids. In fact, it was somewhat inhibitory. At this stage, experiments became quite erratic and often could not be reproduced. The study of amino acids was discontinued. The reasons for the erratic results are not known, but it seems probable that, though the resazurin was always reduced when the media were inoculated, they were not always poised at a low enough $E_h$ to allow rapid growth. Different mixtures of amino acids in the simple basal medium of known composition might have a considerable effect on $E_h$. It is also possible that some of the erratic results were due to amino acid inhibitions and antagonisms.

Excellent and quite reproducible growth was possible in the medium containing ten amino acids. The results of one experiment on the growth obtained in this medium as compared to casein hydrolysate, alanine, phenylalanine, and

cysteine, and cysteine only, is shown in Figure 2. It should be noted that in some experiments growth in the medium containing three amino acids or only cysteine was more comparable to that in media containing casein hydrolysate or ten amino acids.

*Sulfur requirements and reducing agents.* From the results above it was evident that cysteine and/or sulfate could serve as the sole source of sulfur, other than that present in biotin, for growth of the organism. To obtain more information on the sulfur requirements, attempts were made to replace the cysteine with nonsulfur-containing reducing systems. Strain S85 was used. Table 5 shows some of the results obtained in an attempt to replace cysteine with ascorbic acid. It is evident that ascorbic acid would not replace cysteine as reducing agent, even when levels of cysteine too low to reduce the medium by itself (0.01% or less) were present. The 0.01% level of cysteine undoubtedly would meet the sulfur requirements for maximum or at least near-maximum growth. The fact that good growth occurred when 0.05% of cysteine was present in addition to ascorbic acid indicates that ascorbic acid was not inhibitory to the organism. It seems probable that, though the ascorbic acid alone reduced the resazurin in the medium, the $E_h$ was too high to allow initiation of growth.

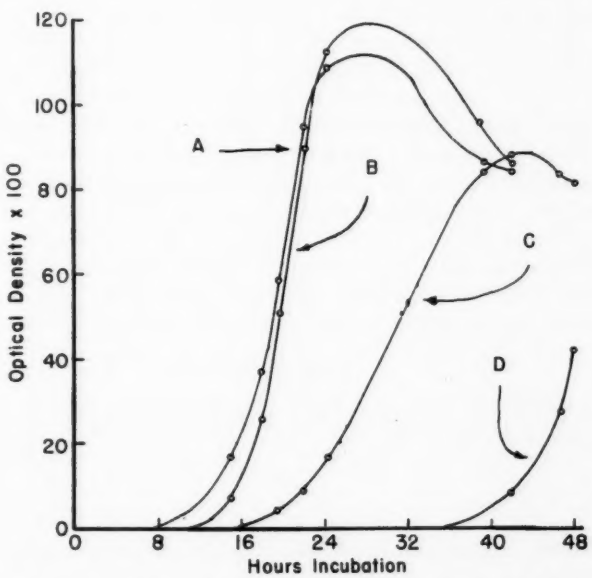


FIG. 2. The effect of different numbers of amino acids on growth of Strain S85. The basal medium was the same as that shown in Table 1, except that casein hydrolysate and all of the B vitamins but PABA and biotin were omitted and the level of cysteine was 50 mg. A, alanine, isoleucine, serine, phenylalanine, tryptophane, glutamic, lysine, arginine, and proline; B, casein hydrolysate; C, alanine and phenylalanine; D, no amino acids other than the cysteine present in the basal medium.

TABLE 5

Effect of some reducing agents and/or sulfur sources on growth of Strain S85[a]

| Ingredients added to basal medium[b] | Growth (O. D. × 100) |
|---|---|
| Experiment 1 | |
| Cysteine · HCl (0.05%) | 105 (22) |
| Ascorbic Acid (0.05 or 0.1%) | 0 (72) |
| Ascorbic Acid (0.05 or 0.1%) + cysteine · HCl (0.05) | 89–105 (22) |
| Ascorbic Acid (0.05 or 0.1%) + cysteine · HCl (0.001, 0.005, or 0.01%) | 0 (72) |
| Experiment 2 | |
| Cysteine · HCl (0.05%) | 101 (24) |
| $Na_2S \cdot 9\ H_2O$ (0.025 or 0.125%) | 110 (24) |
| Experiment 3 | |
| Cysteine · HCl (0.05 or 0.25%) | 102–107 (20) |
| Sodium thioglycolate (0.1 or 0.025%) | 0 (112) |
| Sodium thioglycolate (0.05%) | 13 (112) |

[a] See Footnote [b], Table 2.

[b] The basal medium was the same as Medium A (Figure 2), except that cysteine · HCl was deleted. The reducing agents, ascorbic acid and thioglycolate, were added both before the basal medium was sterilized and after sterilizing. In the latter case, they were sterilized separately, both by autoclave and by glass filter. Similar results were obtained, regardless of the treatment.

An attempt was made to replace the cysteine-reducing system with palladium chloride or palladium black-hydrogen reducing systems similar to those used by Mylroie and Hungate (27) for isolation of sludge methane bacteria. However, in media containing all known constituents the lowest levels of palladium that allowed reduction of the medium (0.001-0.002%) were toxic to the bacterium.

Results in Table 5 show that sodium thioglycolate allowed little growth but that sodium sulfide was quite satisfactory in replacing cysteine as reducing agent.

Data in Table 6 show that cysteine or $Na_2S \cdot 9\ H_2O$ served quite well as the sole source of sulfur for growth. The amounts required as sulfur source could not be determined, because higher levels were necessary to reduce the medium.

An attempt was made to grow the organism in the basal medium (Table 6) with $(NH_4)_2SO_4$ as the sole source of sulfur and no reducing agent added. A large inoculum, 0.5 ml. of unwashed, undiluted cells (O.D. 1.15), was used in the hope that it would reduce the medium, and daily serial transfers could

TABLE 6

Some data on the effect of cysteine and sulfide as the sole source of sulfur on Strain C85[a]

| Ingredients added to basal medium[b] | Growth (O.D. × 100) |
|---|---|
| Experiment 1 | |
| Cysteine + $(NH_4)_2SO_4$[c] | 92 (21) |
| Cysteine + $NH_4Cl$ | 82 (21) |
| Experiment 2 | |
| Cysteine + $(NH_4)_2SO_4$ | 96 (34) |
| Cysteine + $NH_4Cl$ | 77 (34) |
| $Na_2S \cdot 9\ H_2O$ + $NH_4Cl$ | 70 (34) |

[a] See Footnote [b], Table 2.

[b] The basal medium was that used in the study of mineral requirements, except that cysteine, sulfide, and ammonium salts were present as indicated. Minerals added included $KH_2PO_4$, 0.09%; NaCl, 0.09%; $CaCl_2 \cdot 6\ H_2O$, 0.002%; $MgCl_2 \cdot 6\ H_2O$, 0.0016%; $CoCl_2 \cdot 6\ H_2O$, 0.0004%.

[c] Cysteine · HCl · $H_2O$, $(NH_4)_2SO_4$, and $NH_4Cl$ were added at the 0.05%, 0.09%, and 0.077% levels, respectively.

be made to dilute out sulfur compounds in the inoculum. However, reduction of the medium and growth occurred only on the first transfer. On addition of 0.1% ascorbic acid, the same results were obtained.

*Mineral requirements.* It is evident from data in Table 7 that when $PO_4^{---}$, $K^+$, $Mg^{++}$, $NH_3$, or $Ca^{++}$ was deleted from the otherwise complete medium little or no growth occurred. $Fe^{++}$, $Co^{++}$, and $Mn^{++}$ had no effect on growth, indicating that they were not essential or that they were present in the medium as contaminants. About 0.3 mg. of $PO_4^{---}$, 0.2 to 0.3 mg. of $NH_3$, 0.01 mg. $Mg^{++}$, and 0.05 mg. of $Ca^{++}$ per 5 ml. of medium were required for maximum growth (Figures 3 and 4). Whether other cations would spare or replace the $Ca^{++}$

TABLE 7

Effect of deletion of minerals on growth of Strain S85[a]

| Ion deleted | Growth (O.D. × 100) | Ion deleted | Growth (O.D. × 100) |
|---|---|---|---|
| None deleted[b] | 93 (41) | Ammonium | 0 (97) |
| Phosphate[c] | 0 (97) | Calcium | 9 (97) |
| Potassium[d] | 0 (97) | Cobalt | 89 (41) |
| Magnesium | 16 (33) | Manganese | 85 (41) |
| Iron | 85 (41) | | |

[a] See Footnote [b], Table 2.

[b] Minerals added to the basal medium included $KH_2PO_4$, 0.09%; NaCl, 0.09%; $(NH_4)_2SO_4$, 0.09%; $MgSO_4 \cdot 7\ H_2O$, 0.002%; $FeSO_4 \cdot 7\ H_2O$, 0.0005%; $CaCl_2 \cdot 6\ H_2O$, 0.002%; $CoCl_2 \cdot 6\ H_2O$, 0.0004%; and $MnSO_4 \cdot H_2O$, 0.001%.

[c] KCl, 0.056%, was added in place of $KH_2PO_4$.

[d] $NaH_2PO_4 \cdot H_2O$, 0.09%, was added in place of $KH_2PO_4$.

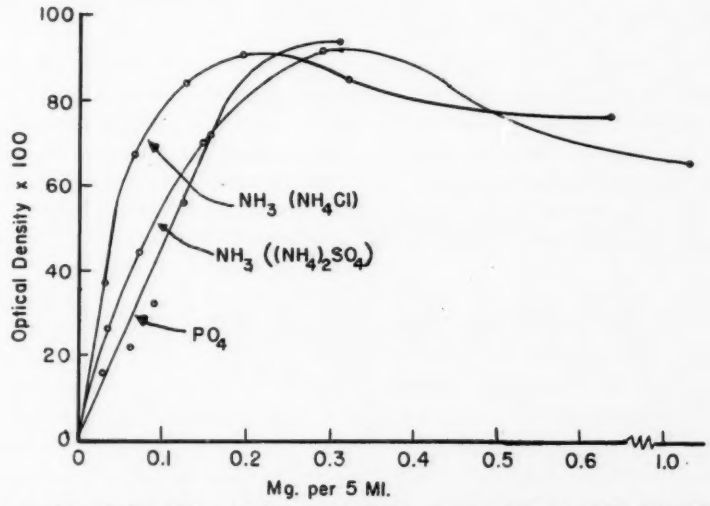


FIG. 3. The levels of $PO_4$ and $NH_3$ required for growth of Strain S85. Minerals added to all three media included NaCl, 0.09%; $MgCl_2 \cdot 6\ H_2O$, 0.0016%; $CaCl_2 \cdot 6\ H_2O$, 0.002%, and $CoCl_2 \cdot 6\ H_2O$, 0.0004%. The $PO_4$-deficient medium contained $(NH_4)_2SO_4$, 0.09%; KCl, 0.056%; and $PO_4$ was added as $NaH_2PO_4 \cdot H_2O$. The $NH_3$-deficient media contained $KH_2PO_4$, 0.09% and, when $NH_3$ was added as $NH_4Cl$, 0.09% of $Na_2SO_4$ was included.

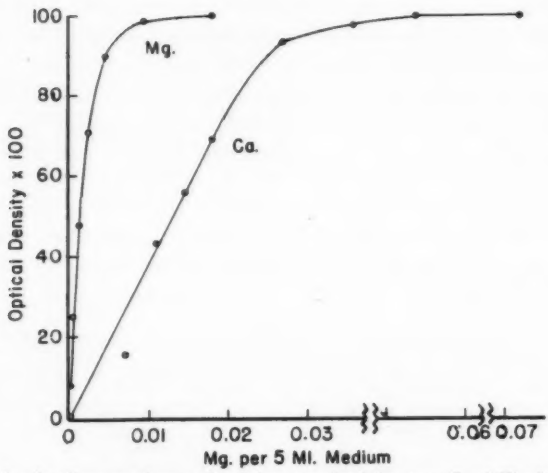


FIG. 4. The levels of magnesium and calcium required for growth of Strain S85. Minerals added to the basal medium included $KH_2PO_4$, 0.09%; $(NH_4)_2SO_4$, 0.09%; NaCl, 0.09%, and $CoCl_2 \cdot 6\,H_2O$, 0.0004%. $CaCl_2$, 0.001%, and $MgCl_2 \cdot 6\,H_2O$, 0.0016%, were added to the Mg- and Ca-deficient media, respectively, and the same salts were utilized in determining levels required.

or $Mg^{++}$ was not determined. However, it was evident that both $Ca^{++}$ and $Mg^{++}$ were required in the presence of the other and that $Fe^{++}$, $Co^{++}$, or $Mn^{++}$ would not replace them in the low levels used.

It was advantageous to include the low level of $Co^{++}$ because the medium reduced at a much slower rate when reducing agent was added without it. Five p.p.m. of $Co^{++}$ inhibited growth and 15 p.p.m. completely stopped growth.

It was evident (Table 8) that $K^+$ was essential for growth. The 0.082 *N* level of $Na^+$ was the lowest added to the medium equilibrated with the 80% $CO_2$ gas, due to the necessity of adding alkali to adjust the pH. At this level of $Na^+$, best growth occurred at about 0.01 *N* $K^+$. At the 0.098 *N* level of $Na^+$, the amount

TABLE 8

Effect of different levels of potassium and sodium on growth of Strain S85 in potassium-free medium[a]

| | Growth (O. D. × 100) | | | |
|---|---|---|---|---|
| Normality $Na^+$ | 0.082 | 0.098 | 0.164 | 0.328 |
| Normality $K^+$ | | | | |
| 0.05 | 48 (24)[b] | 30 (24) | 2 (19) | 0 (48) |
| 0.01 | 85 (22) | 74 (24) | 19 (22) | 0 (48) |
| 0.003 | 64 (22) | 92 (24) | 24 (24) | 0 (48) |
| 0.001 | 3 (19) | 0 (48) | 2 (19) | 0 (48) |
| 0.0003 | 0 (48) | 0 (48) | 0 (48) | 0 (48) |

[a] Minerals added to the basal medium included $(NH_4)_2SO_4$, 0.09%; $NaH_2PO_4 \cdot H_2O$, 0.09%; $CoCl_2 \cdot 6\,H_2O$, 0.0004%; $CaCl_2$, 0.001%; $MgCl_2 \cdot 6\,H_2O$, 0.0016%, and K was added as KCl.
[b] See Footnote [b], Table 2.

regularly added to media, the best growth occurred with 0.003 *N* $K^+$. At higher levels of $Na^+$, growth was poor, regardless of the $K^+$ level.

When sodium-free medium equilibrated with 80% $CO_2$ was prepared with $K_2CO_3$ and KOH substituting for the sodium compounds, very poor growth occurred at the lowest level of $K^+$ (0.082 *N*) and none occurred at the 0.164 *N* level. At the 0.082 *N* level of $K^+$, maximum O.D. was 0, 4, 21, and 0 for 0.3, 0.1, 0.03, and 0.01 *N* $Na^+$, respectively.

To study the effect of wider ranges of $Na^+$ and $K^+$ concentrations, the $CO_2$ in the gaseous phase of the basal medium was lowered by using a gas mixture containing 10% $CO_2$ and 90% $N_2$, and the $Na_2CO_3$ concentration was lowered to 0.12%. Growth in this basal medium was rather poor and erratic, probably because of the high bicarbonate requirement of the organism (9) and the lowered buffer capacity of the medium. Table 9 shows results of one of the better experiments. Growth was very poor at the lowest level of $Na^+$ (0.029 *N*), regardless of the $K^+$ level added, indicating that $Na^+$ was essential for growth. The results

TABLE 9

Effect of different levels of potassium and sodium on growth of Strain S85 in potassium-free basal medium with 10% $CO_2$—90% $N_2$ gaseous phase[a]

| | Growth (O. D. × 100) | | | | |
|---|---|---|---|---|---|
| Normality $Na^+$ | 0.029 | 0.057 | 0.084 | 0.111 | 0.152 |
| Normality $K^+$ | | | | | |
| 0.15 | 0 (40) | 0 (40) | 0 (40) | ..... | ..... |
| 0.10 | 4 (40) | 0 (40) | 0 (40) | 0 (40) | ..... |
| 0.05 | 1 (16) | 44 (31) | 20 (27) | 5 (27) | 0 (40) |
| 0.01 | 0 (40) | 3 (31) | 65 (27) | 24 (24) | 4 (16) |
| 0.003 | 0 (40) | 2 (31) | 30 (24) | 46 (27) | 4 (16) |
| 0.001 | 0 (40) | 2 (31) | 20 (27) | 9 (27) | 0 (40) |
| 0.0003 | 0 (40) | 0 (40) | 0 (40) | 0 (40) | 0 (40) |

[a] See footnotes of Table 8.

again demonstrated the essentiality of $K^+$. Best growth occurred with the 0.05, 0.01, and 0.003 *N* $K^+$ when 0.057, 0.084, and 0.111 *N* $Na^+$ levels, respectively, were present. Thus, at lower levels of sodium, higher levels of potassium were necessary for best growth and the reverse was also true.

The organism could be grown in a medium with sulfate salts replacing all chlorides and cysteine (free base) replacing cysteine · HCl · $H_2O$.

*Miscellaneous experiments.* To determine if *B. succinogenes* would digest cellulose in a known medium containing few ingredients, Strain S85 was inoculated into Medium C of Figure 2 modified to contain approximately 0.2% of thoroughly washed filter paper cellulose (9) in place of the glucose. The organism was carried on this medium through six daily transfers, using 0.1 ml. inoculum into 10 ml. of medium. After 24-hr. incubation of each tube a trace to about one-third of the cellulose had visibly disappeared. After 48 hr. practically all had disappeared. Thus, though considerably slower than in more complex media, growth and cellulose digestion occur in the simple, chemically defined medium with cellulose replacing glucose.

TABLE 10

Effect of various mixtures of volatile fatty acids on the growth of Strain S85[a]

| Volatile fatty acid added | Casein hydrolysate | Growth (O. D. × 100) |
|---|---|---|
| | Experiment 1 | |
| All[b] | Present | 117 (20) |
| All except acetate | Present | 122 (20) |
| Valerate + isovalerate | Present | 122 (18) |
| Valerate | Present | 0 (96) |
| Isovalerate | Present | 1 (96) |
| None | Present | 0 (96) |
| | Experiment 2 | |
| All | Present | 107 (23) |
| All | Deleted | 117 (71) |
| Valerate + isovalerate | Present | 105 (23) |
| Valerate + isovalerate | Deleted | 85 (63) |

[a] See Footnote [b], Table 2. The basal medium was that shown in table, except that only biotin and PABA of the B vitamins were added and the volatile fatty acids and casein hydrolysate were deleted.

[b] The fatty acids were added in the following concentrations ($\mu M$/100 ml.): acetate, 420; propionate, 150; *n*-butyrate, 100; isobutyrate, isovalerate, and DL-α-methyl-*n*-butyrate, 30 of each; and *n*-valerate, 60.

The results shown in Table 10 indicate that Strain S85 had not lost its two component volatile fatty acid requirements after being maintained in the laboratory for over 5 yr. and, contrary to studies on Strain C94 of *Ruminococcus flavefaciens* (2), acetate was not stimulatory. Also, the presence of the complete mixture of ruminal volatile fatty acids did not significantly stimulate growth above that obtained with only valerate and isovalerate, and this was true both in the presence and absence of amino acids.

## DISCUSSION

In preliminary studies on vitamin requirements of Strain S85, the essentiality of PABA as well as biotin was established. None of the other B vitamins or a mixture of purines and pyrimidines (10) would replace the PABA requirement. It thus seems evident that this strain changed somewhat during cultivation on artificial medium, since PABA had no effect on growth of Strain S85, or two other strains, in the present study.

The results indicated that folic acid would not replace the PABA required for good growth of Strains S61 and CB40.

Whether biotin could be replaced by materials such as desthiobiotin, pimelic acid, or oleic acid was not determined.

The activity of biotin and PABA as growth factors for *B. succinogenes* seems to substantiate the results of Bentley *et al.* (6), who found biotin and PABA to be active in promoting cellulose digestion by washed suspensions of the mixed ruminal flora. It is of interest to speculate that the method used enriched the *B. succinogenes* of the mixture because only PABA and biotin of the B vitamins stimulated cellulose digestion. Other workers using the mixed ruminal flora have found other vitamins to be stimulatory to cellulose digestion (22, 25). Also, work on other species of ruminal cellulolytic bacteria (5, 11,

16, 17) indicate that these required some B vitamin other than, or in addition to, biotin and PABA.

It is of interest that biotin and PABA also were shown to satisfy the B vitamin requirements of some noncellulolytic ruminal anaerobes, i.e., a strain of *Lachnospira multiparus* (15) and a strain of *Bacteroides ruminicola* subsp. *brevis* (12). The latter organism is similar to *B. succinogenes* in many respects, including morphology and fermentation products, but differs in many respects, e.g., it had no volatile acid requirement but did require enzymatic hydrolysate of casein.

The very simple nitrogen requirements of *B. succinogenes*, $NH_3$ being the only essential nitrogenous compound, appear to be similar to those of several other ruminal bacteria. Gill and King (17) found no essential amino acids for growth of a strain of *Butyrivibrio*. Large amounts of $NH_3$ were assimilated, and the amount assimilated was independent of the amount of amino acid in the medium. None of the amino acids were essential for growth of *Streptococcus bovis* (28) or *L. multiparus* (15). Data of Allison (1) indicate that Strain C94 of *R. flavefaciens* will grow in a medium with $NH_3$ as the only source of nitrogen.

It is of considerable interest that Allison, Bryant, and Doetsch (3) found that this strain did not incorporate an appreciable amount of preformed amino acids into cellular protein during growth, but did utilize isovalerate in the synthesis of leucine. The results made it appear probable that the essentiality of isovalerate as a growth factor for *R. flavefaciens* was due to the organism's inability to assimilate preformed amino acids and its inability to synthesize the branch-chained carbon skeleton of leucine from compounds other than the branch-chained volatile acids.

It seems probable that a similar situation exists in *B. succinogenes*, which has been shown to require one of certain branch-chained, and one of certain straight-chained, volatile fatty acids for growth (10). The facts, that ammonia is essential and no amino acids are essential, tend to support this probability.

The possibility exists that the stimulatory effect of amino acids on the initiation of growth of *B. succinogenes* as shown in the present work, and on growth of a strain of *R. albus* (16) and two strains of *R. flavefaciens* (1, 5), may be due to factors not related to their incorporation into cellular protein. Among these factors might be an effect on the $E_h$ of the culture medium or their ability to antagonize inhibitory effects of other compounds or ions in the medium.

It also is possible that ruminal organisms such as *B. ruminicola* subsp. *brevis*, which appear to require amino acids for growth (12), do not incorporate them directly into cellular protein but produce volatile fatty acids which are then used to synthesize amino acids incorporated into cellular protein. This organism is known to actively produce ammonia from protein hydrolysates. It would be of interest to determine if this organism can grow in media without amino acids if ammonia, and ruminal volatile fatty acids, were present.

Further studies on the nitrogen requirements of *B. succinogenes* and other ruminal bacteria should bring to light information regarding the ruminal metabolism of protein. It seems evident that work on nutrient requirements for growth,

as done in the present study, is not enough. Of equal importance is the determination of the actual nutrients utilized by the organism to synthesize cellular constituents, as done in studies such as those of Warner (30) and of Allison, Bryant, and Doetsch (3).

Ruminal bacteria may well be very useful tools for use in furthering basic knowledge of mechanisms involved in amino acid assimilation and synthesis of amino acids and protein.

A detailed study of the sulfur requirements of *B. succinogenes* was not possible under the conditions studied, because the only reducing agents found to be satisfactory for growth of the organism also served as sole sources of sulfur, and the level required as reducing agent was almost certainly higher than the amount needed as sulfur source.

The fact that sulfide can serve as the sole source of sulfur suggests that the organism is well adapted to life in the rumen, where sulfide appears to be the main source of sulfur that is continually present in a form available to bacteria for assimilation. It is known that appreciable amounts of sulfide usually are present in the rumen, and it is believed that the bulk of the sulfur of the rumen passes through a stage where it is present as sulfide (4, 23). Lewis (24) was unable to detect sulfur-containing amino acids in cell-free rumen fluid, though some other amino acids were detected, especially shortly after the animals were fed. It is now well established that sulfate is rapidly reduced to sulfide (23) and that cysteine and cystine is broken down with a release of sulfide (24, 29).

The fact that cysteine serves as an excellent source of sulfur for *B. succinogenes* [it does not produce sulfide from cysteine or sulfate (9)], plus the fact that sulfide is produced from cysteine or cystine by the mixed flora, seems to contradict the data of Hunt *et al.* (21), who found that cystine was a poor source of sulfur as indicated by in vitro cellulose digestion by the mixed ruminal flora. This finding might have been due to the possibility that cystine would tend to raise the $E_h$ of the system to a point where the activity of the cellulolytic anaerobes was depressed.

Emery, Smith, and Fai To (15) studied a number of strains of ruminal bacteria for $S^{35}O_4$ incorporation during growth in complex medium. While some species showed a significant incorporation and others, none, the data on Strain S85, *B. succinogenes*, were inconclusive.

In the present work, the experiments in which attempts were made to grow the organism from large inocula in media with sulfate as the sole source of sulfur indicate that sulfate can not serve as the sole source of sulfur and/or that even large numbers of cells of the organism are unable to lower the $E_h$ of the medium enough for growth initiation. Although neither of these points has been proved, it is almost certain that the latter assumption is true. For instance, if the organism were grown in a good medium until growth were about O.D. 0.4-0.6, and then a small amount of air admitted to oxidize the medium, growth would not resume. In work in progress on the cellulolytic cocci, similar observations have been made in strains which previous studies showed would not produce hydrogen (13). Strains which produce hydrogen appear to be much less sensitive to changes in $E_h$, once growth has been initiated.

The growth requirement of *B. succinogenes* for $Mg^{++}$ and $Ca^{++}$, and the fact that addition of $Mn^{++}$, $Cu^{++}$, $Co^{++}$, and $Fe^{++}$ was neither essential nor stimulatory, appear to be in agreement with the results of Hubbert, Cheng, and Burroughs (18), who found $Ca^{++}$ and $Mg^{++}$ to be slightly stimulatory to cellulose digestion by the mixed ruminal flora. The results make it seem probable that at least part of the effect of $Ca^{++}$ noted by the above workers, and the in vivo stimulation of cellulose digestion by $Ca^{++}$ noted by White *et al.* (31) in sheep receiving rations containing 5 % corn oil, was due to its effect on growth of the cellulolytic bacteria.

The fact that $Na^+$ in addition to $K^+$ was essential for growth of *B. succinogenes,* and the fact that higher levels of $Na^+$ did not increase the organisms' requirement for $K^+$, do not appear to be in agreement with data obtained on cellulose digestion by the mixed ruminal flora in vitro (19). However, the latter phenomenon may have been obscured by the apparently great sensitivity of the organism to variations in salt concentration.

The fact that at lower levels of $Na^+$ higher levels of $K^+$ were necessary for best growth might suggest a sparing action of one of these ions on the other. However, it is also possible that this was due to the effect of the sodium and potassium chlorides on osmotic pressure. The organism never exhibited appreciable growth when the calculated total ion concentration was outside the range of about a 0.6 to 1.2% solution of NaCl.

The essentiality of $Na^+$ is of considerable interest, as only certain marine bacteria and a halophile, among bacteria so far studied, have shown a need for this ion [see MacLeod and Onofrey (26)]. It is also of interest that the amounts of $Na^+$ and $K^+$ necessary for best growth of Strain S85 are similar to those of marine bacteria (26) and certain types of mammalian cells (14).

The present results indicate the relatively great ability of *B. succinogenes* to synthesize cellular constituents. The nutrition of this organism including the relatively high requirement for $CO_2$ (9), the requirement for volatile fatty acids (10), the essentiality of $NH_3$, and nonessentiality of amino acids, the ability to utilize sulfide as the sole source of sulfur, and the essentiality of $Na^+$, indicate how well this cellulolytic bacterium is adapted to the environment of the rumen.

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# ACTIVATION AND CELL-SURFACE LOCALIZATION OF CERTAIN β-GLUCOSIDASES OF THE RUMINAL FLORA

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**SUMMARY**

Kinetic and inhibition studies of the cellulases in crude extracts of ruminal ingesta indicated the participation of sulfhydryl groups and polyvalent metal ions in enzymic hydrolysis of cellulose. As a result, the assay substrate has been modified by inclusion of ferric chloride. A minimum estimate was obtained of the proportion of the cellulolytic activity in ruminal ingesta which is intimately associated with bacterial cell surfaces. A series of fractionations employing washing of the bacterial cells by centrifugation, rupture in a French pressure cell, and lysis using desoxycholate yielded an estimate of 5%. Reasons for suspecting that surface-bound cellulases may occur in considerably larger proportions were discussed.

Microscopic examination of decomposing cellulosic materials freqently reveals numerous bacterial cells concentrated in the pits and crevices which appear on the surfaces of the fibers early in the course of degradation. This observation has been interpreted as presumptive evidence that intimate contact between the fiber and the cell is essential to solubilization of the substrate, perhaps as a consequence of surface-bound cellulases which can not function as freely diffusing enzymes. This interpretation, although provocative in connection with the energy economy of the cells, is highly speculative; and direct experimental evidence that certain microbial cellulases are intimately associated with the cell surface has not appeared.

The results of investigations of the localization of the β-glucosidases which initiate certain phases of the fermentation of feedstuff in ruminants have been contradictory. Kitts and Underkofler (7) initially reported that the cellulases of the rumen appeared to be localized on microbial cell surfaces. Conflicting observations have appeared from this laboratory, Gill and King (2, 3), and certain of the properties of the extracellular ruminal cellulases have been described, King (6). As a result of these latter investigations, it is evident that typical, freely diffusing, extracellular enzymes are an important component of the cellulase complex which functions in the ruminal fermentation. The substance of the present report concerns direct evidence that surface-bound β-glucosidases do function in the ruminal microflora and that sulfhydryl groups and metal ions are involved in the action of certain ruminal β-glucosidases.

## EXPERIMENTAL METHODS

Assays of β-glucosidase were conducted viscosimetrically, King (6), and by measurement of reducing sugar liberation, Hash and King (5). In both assays the substrate was modified by including $10^{-3}$ *M* $FeCl_3$.

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In investigating the participation of sulfhydryl groups and metal ion activation of the ruminal β-glucosidases, Fraction 1, King (6), was used as the enzyme source. This fraction consisted of those mixed enzymes which were most rapidly eluted from whole ruminal ingesta by dilute phosphate buffer. After dialysis against running tap water, the preparation was lyophilized and stored over $CaCl_2$ at −18° C. When resuspended in dilute buffers a small amount of insoluble material remained. This residue was removed by centrifugation.

In studying localization of the enzymes on bacterial cell surfaces, the material was obtained par fistulum from steers fed a poor-quality clover–timothy hay mixture supplemented with from 2 to 4 lb. of grain concentrate per day. Samples were obtained from the lower portion of the hay mat, Smith *et al.* (11), approximately 1 hr. after the morning feeding. After squeezing the ingesta through four layers of cheese cloth, the liquid portion, hereafter referred to as rumen fluid, was taken to the laboratory in completely filled bottles.

An initial centrifugation at 1,000 × G for 10 min. at 0° C. yielded a supernatant from which most of the plant debris and protozoal cells had been removed. Further sedimentation of this supernatant at 25,000 × G for 30 min. at 0° C. packed the mixed bacterial cells tightly and resulted in a supernatant essentially free of bacteria. By careful resuspension of the upper layers of the pellet, preparations were obtained which contained neither recognizable plant particles nor protozoa in appreciable amounts. Morphologically, these suspensions were very heterogeneous, however. As a result, some question must remain concerning the absolute absence of plant residues. Gram-negative forms predominated.

Lysis of the cells was conducted in 0.05 *M* tris(hydroxymethyl)amino-methane-maleate buffer at pH 6.8 containing 50 mg. of sodium desoxycholate per milliliter, using a 15-min. treatment at room temperature (35 to 40° C.). Under these conditions, approximately 50% of the cells were lysed as estimated by the decrease in turbidity corrected for the release of quite large amounts of soluble pigments during lysis. Longer periods of treatment did not increase the amount of lysis appreciably.

Mechanical rupture of the cells was done in a French pressure cell at 12,000 p.s.i., collecting the fragmented cells in a chilled beaker. Microscopic examination of smears stained with nigrosin after a single treatment indicated fragmentation of more than 90% of the cells. Accurate estimation of the efficiency of fragmentation was not attempted because of the morphological heterogeneity of the original cell suspensions.

## RESULTS AND DISCUSSION

Observation that the amount of glucose produced enzymically from carboxymethylcellulose decreased at increasing dilutions of the enzyme, when the incubation times were increased in proportion to the dilution of the enzyme, indicated a cofactor requirement. Several potential inhibitors and activators were evaluated, with the results shown in Table 1. Involvement of sulfhydryl groups is suggested by these data, but the relative stability of the enzymes to oxygen,

TABLE 1

Inhibition and stimulation of crude ruminal cellulases[a]

| Test compound | Activity as per cent of unsupplemented control | Test compound | Activity as per cent of unsupplemented control |
|---|---|---|---|
| None | 100 | $K_2S_2O_3$ | 74 |
| Metallo-enzyme inhibitors | | $MnCl_2$ | 85 |
| NaCN | 36 | $NH_2OH \cdot HCl$ | 81 |
| $NaN_3$ | 99 | $NaHAsO_3$ | 82 |
| α,α-Bipyridyl | 66 | $FeSO_4$ | 100 |
| *o*-Phenanthroline | 100 | Bovine serum albumin | 100 |
| Sodium EDTA | 94 | $MgCl_2$ | 106 |
| Sulfhydryl inhibitors | | $CaCl_2$ | 111 |
| *p*-CMB | 88 | $Li_2SO_4$ | 116 |
| Iodoacetamide | 57 | KCl | 117 |
| $HgCl_2$ | 21 | $BaCl_2$ | 122 |
| $AgNO_3$ | 86 | $SnCl_2$ | 124 |
| $Pb_2(C_2H_3O_4)_2$ | 95 | Cysteine | 132 |
| Other compounds | | KI | 132 |
| $UO_2(NO_3)_2$ | 75 | $Na_2HAsO_4$ | 132 |
| $Na_2S$ | 35 | $FeCl_3$ | 138 |
| $ZnCl_2$ | 96 | $COCl_2$ | 141 |
| $CuCl_2$ | 70 | | |

[a] Reaction mixtures consisted of 0.5 ml. of enzyme (0.5 mg. of crude powder) in water, 0.5 ml. of water or test compound (final concentration = $10^{-3}$ *M*), plus 1.0 ml. of assay substrate. Incubation was at 37° C. for 2 hr. Activity was measured in terms of glucose equivalents produced per tube, corrected for both the heat-inactivated enzyme control and for the color produced by each test material in the absence of enzyme.

Gill and King (2), implies that the sulfhydryl groups are somewhat protected. Basu and Whitaker (1) reported a similar sensitivity of purified cellulase from *Myrothecium verrucaria* to inhibitors of sulfhydryl-dependent enzymes. The stimulatory effect of serum albumin which Whitaker (14) reported was not evident in the present system, perhaps because only soluble substrates have been used in the present study.

The inhibitory action of cyanide, ethylenediaminetetraacetate, and α,α-bipyridyl, and the apparent activation by ferric and cobaltous ions seen in Table 1 were examined further. Both $CoCl_2$ and $FeCl_3$ reversed the inhibitions by ethylenediaminetetraacetate and α,α-bipyridyl at $10^{-3}$ *M*. In addition, the data in Figure 1 demonstrate that $FeCl_3$ alone prevented the decrease in product formation as decreasing concentrations of enzyme were incubated for inversely proportional times. The specificity of the metal ion requirement has not been pursued further, since methods for separating the several enzymic components known to be present in the crude preparation have not been developed. All subsequent assays, however, were carried out in reaction mixtures containing $10^{-3}$ *M* $FeCl_3$, there being no information bearing on what proportion of the ruminal cellulases is activated by trivalent iron and no indication of inhibition by iron.

Using the viscosimetric assay, readily detectable amounts of enzyme were observed in whole cell suspensions washed as many as eight times in large volumes of either 0.05 *M* phosphate or tris(hydroxymethyl)aminomethane-maleate buffer at pH 6.8. In a typical experiment, 8.0 ml. of rumen fluid was subjected to the low- and high-speed centrifugations described earlier. After discarding the supernatant from the high-speed centrifugation, the pellet was resuspended in 8.0 ml.

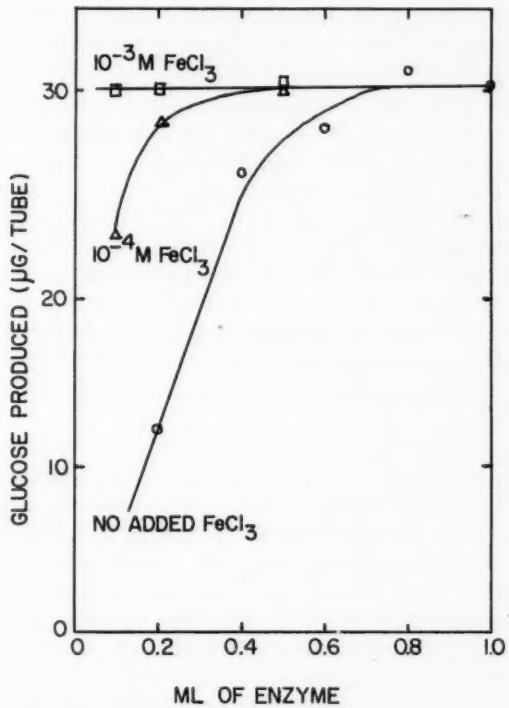


FIG. 1. Reversal by iron of the reduction in enzyme activity as a result of dilution. The concentration of enzyme (milligram/milliliter) times the time of incubation (minutes) in all instances = 30 mg.-min. per milliliter.

of buffer and centrifuged at 25,000 × G for 30 min. at 0° C. The resulting supernatant was then assayed. This washing procedure was repeated three more times, assaying each supernatant and the final pellet. The total recovery of enzyme in the successive supernatants was 175, 145, 44, and 30 units, and in the final resuspended whole cells, 122 units. With a second, similar sample of rumen fluid the successive washes contained 88, 26, 30, and 17 units, and the cells contained 131 units.

Microscopic examination of the washed cell suspensions indicated the presence of some plant residues having centrifugal properties similar to those of the bacteria. The possibility existed that the activity of the washed cells represented enzymes adsorbed on the cellulosic residues, the enzymes being desorbed when added to the soluble assay substrate. That this was not so was indicated by control experiments, in which parallel cell preparations were made using assay substrate as the washing medium in one preparation and the assay buffer components as the washing medium in the other. In a typical experiment involving four washing steps, the buffer-washed cells retained 138 units of enzyme com-

pared to 145 units in the cells washed with carboxymethylcellulose, the difference being insignificant. These results indicate that the activity is associated with the bacterial cells themselves.

If the activity of washed cell suspensions were attributable to localization of the enzymes on the surface of the cells, two observations should be possible. First, lysis of the cells should be associated with release of the enzymes into solution, although either incompleteness in solubilization of the cell wall or denaturation of the enzyme might cause the quantitative correlation between lysis of cells and solubilization of enzyme to be approximate. Second, nonlytic fragmentation of the cells using the French pressure cell should yield enzymically active particles (cell wall fragments) from which the enzyme could be released later by lysis. As a corollary, the amount of enzyme retained by the cell wall fragments after treatment in the pressure cell but solubilized by subsequent lysis should serve as a minimum estimate of the amount of surface-localized cellulase in the ruminal flora.

Estimating lysis turbidimetrically after 1-hr. incubation periods, less than 5% lysis was obtained using lysozyme (2 to 200 $\gamma$/ml) alone, lysozyme followed by treatment with $NH_4OH$, lysozyme following pretreatment of the cells with acetone, Warren *et al.* (12), or lysozyme in conjunction with ethylenediaminetetraacetate, Repaske (10), 0.25 *M* glycine, 0.25 *M* NaCl, or 0.5 mg/ml of trypsin. Cells suspended at 0° C. for 15 min. in saturated NaCl, harvested by centrifugation, and then resuspended in distilled water likewise failed to lyse significantly. Polymyxin-B sulfate at concentrations between 8.25 and 125 $\gamma$/ml, followed by treatment with lysozyme at concentrations of 3 to 30 $\gamma$/ml, as described by Warren *et al.* (13), failed to produce appreciable lysis. Saponin, bile salts, and sodium-desoxycholate lysed the preparations rapidly. It was also found that under the conditions used here desoxycholate did not influence the amount of soluble ruminal cellulase adsorbed onto powdered cellulose.

A whole cell suspension was prepared from 64 ml. of rumen fluid as described previously and washed three times in 8 ml. of 0.05 *M* tris(hydroxymethyl)aminomethane-maleate at pH 6.75. Use of this buffer was dictated by the fact that on mixing phosphate and sodium desoxycholate, a thixotropic gel which invalidated viscosimetric assays was formed. The cells were resuspended in 20 ml. of the same buffer and filtered through glass wool to remove poorly dispersed clumps. One-milliliter portions of the cell suspension were then incubated for 15 min. at room temperature with 1.0 ml. of sodium desoxycholate at several concentrations between 0 and 75 mg/ml. Initial and final turbidity measurements were recorded, using the Klett-Summerson colorimeter with the blue filter. After centrifugation at 29,000 $\times$ G for 20 min. in the cold, the supernatants were assayed viscosimetrically for cellulase and the optical density at 380 m$\mu$ was measured in a Spectronic-20 colorimeter and in the Klett-Summerson colorimeter using the blue filter. At this wave length absorption by the post-lysis, supernatant was maximum. The optical density data using the two instruments were proportional. To correct for the release of soluble pigments

during lysis, the per cent lysis was calculated as follows: per cent lysis = 100

$$\left[\frac{\text{O.D. before lysis} - (\text{O.D. after lysis} + \text{O.D. of the supernatant})}{\text{O.D. before lysis}}\right]$$

The results are summarized in Figure 2. It is evident that the amount of enzyme solubilized during lysis of the cells was closely related to the amount of lysis. Assay of the original cell suspension indicated 10.8 units of enzyme per milliliter. Those preparations lysed with 50–75 mg. of desoxycholate per milliliter yielded supernatants containing 42.8 to 46.4 units of enzyme per milliliter. The increase in activity as a result of lysis does not appear to be an artifact and will be referred to later.

Assessment of the quantitative significance of the cell-bound cellulases was attempted. In this experiment, the amount of enzyme in each fraction at each step was determined by assay. A 400-ml. portion of rumen fluid was centrifuged at 1,000 × G for 10 min. at 0° C. The resulting supernatant was then centrifuged at 25,000 × G for 30 min. The resulting pellet was resuspended in 50 ml. of 0.05 *M* tris(hydroxymethyl)aminomethane-maleate at pH 6.75 and washed by a second high-speed centrifugation. Washing of the pellet was repeated four more times. Half of the final cell suspension was then lysed under the usual conditions in 50 mg. of sodium desoxycholate per milliliter. The lysed suspension was then centrifuged at high speed, yielding a cell-free supernatant and a particulate residue. The other half of the final cell suspension was treated in a French pressure cell and separated into soluble and particulate components

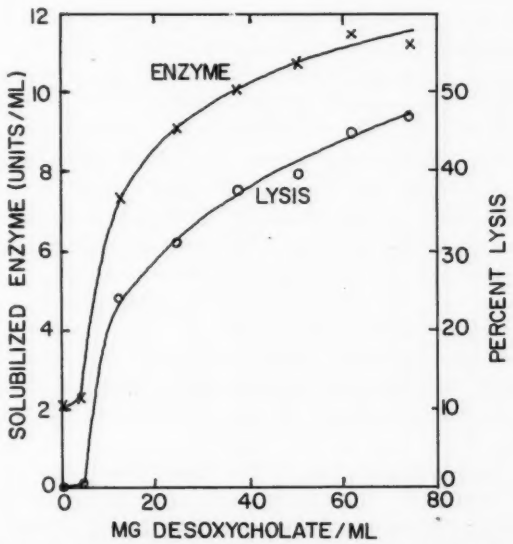


FIG. 2. Lysis and solubilization of particulate enzymes as a result of treatment with desoxycholate.

by high-speed centrifugation. The particulate fraction was resuspended in buffer, lysed using 50 mg. of sodium desoxycholate per milliliter, and centrifuged again at high speed. The results are summarized in Table 2.

The assumption was made that the activity retained in the particulate fraction after treatment in the pressure cell, and later released in soluble form as a result of lysis, gives a minimum estimate of the surface-bound bacterial cellulase. This fraction, the supernatant of Step 10, accounts for approximately 5% of the total activity in the original sample. Several factors indicate that this value is a minimum estimate. In preparing the cell suspensions, emphasis was placed on elimination of plant residues and protozoa rather than on quantitative recovery of bacteria. Some degree of lysis is likely to have occurred during preparation of the cell suspensions. Lysis of the cell suspensions, using desoxycholate at a concentration of 50 mg/ml, was only approximately 50% complete.

The quantitative discrepancies which appear in this experiment can be explained in certain instances. Reliable corrections, however, can not be made because of existing gaps in specific information concerning the enzyme system under study.

The occurrence in ruminal ingesta of substances inhibitory to cellulases has been encountered repeatedly in this laboratory, King (6). Very little is known of either the chemical or the enzymological properties of these inhibitors. The high recoveries seen in the early centrifugal steps (2 through 6) of the experiment summarized in Table 2 probably resulted in part from progressive removal of inhibitors and in part from partial lysis with release of soluble intracellular enzymes.

The increase in the amount of assayable cellulase (Figure 2) which accompanied lysis with desoxycholate may also bear on the excessive recoveries in Steps 2 through 6. A truly intracellular $\beta$-glucosidase which is capable of degrading cellulosic substrates of relatively high degree of polymerization has been described by Youatt (15). Considering the extreme heterogeneity of the cellulolytic ruminal flora, participation of similar intracellular enzymes in the utilization of cellulose as a source of energy must be entertained as a possibility. No measure of the amount of lysis which occurred in the early stages of the fractionation was made. The unusual resistance of the final cell suspensions to lytic processes, however, suggests that many fragile cells may have lysed during washing of the cells, thus releasing normally intracellular $\beta$-glucosidases into the suspending medium.

It is probable that the assay values in Steps 9 and 11, and of the supernatants in Steps 10 and 12, were approximately 10% low, because control experiments showed that desoxycholate was inhibitory to that extent under the conditions used.

Caution in rigid interpretation of the quantitative aspects of the data in Table 2 is also dictated by the fact that the assay measures the activity of a group of enzymes differing in physical properties, specificity, and mode of action, Reese (9), King (6), Grimes *et al.* (4), Hash and King (5), and Miller and Blum (8). The validity of the assay was established for the extracellular

TABLE 2

Fractionation of ruminal cellulases by centrifugation, lysis, and rupture in a pressure cell to access the proportion which is surface-bound

| Step No. | Treatment[a] | Fraction | Use (refer to Column 1) | Total activity[b] (units of cellulase) | Per cent recovery for each step[c] |
|---|---|---|---|---|---|
| 0 | None | Original sample | Step 1 | 16,000 | ..... |
| 1 | Low-speed centrifugation | Sediment | Discarded | 2,930 | |
| | | Supernatant | Step 2 | 12,400 | 96 |
| 2 | High-speed centrifugation | Sediment | Step 3 | 2,700 | |
| | | Supernatant | Discarded | 16,200 | 152 |
| 3 | High-speed centrifugation | Sediment | Step 4 | 2,250 | |
| | | Supernatant | Discarded | 395 | 98 |
| 4 | High-speed centrifugation | Sediment | Step 5 | 2,770 | |
| | | Supernatant | Discarded | 472 | 144 |
| 5 | High-speed centrifugation | Sediment | Step 6 | 2,460 | |
| | | Supernatant | Discarded | 635 | 112 |
| 6 | High-speed centrifugation | Sediment | Steps 7 and 11 | 2,340 | |
| | | Supernatant | Discarded | 222 | 104 |
| 7 | Rupture in pressure cell | ..... | Step 8 | 4,080 | 174 |
| 8 | High-speed centrifugation | Sediment | Step 9 | 1,350 | |
| | | Supernatant | Discarded | 3,470 | 118 |
| 9 | Lysis with desoxycholate | ..... | Step 10 | 1,450 | 107 |
| 10 | High-speed centrifugation | Sediment | Discarded | 618 | |
| | | Supernatant | Discarded | 842 | 101 |
| 11 | Lysis with desoxycholate | ..... | Step 12 | 5,470 | 234 |
| 12 | High-speed centrifugation | Sediment | Discarded | 1,010 | |
| | | Supernatant | Discarded | 3,990 | 92 |

[a] Low-speed centrifugation was carried out at 1,000 × G for 10 min. at 0° C.; high-speed centrifugations were carried out at 25,000 × G for 30 min. at 0° C.

[b] All values have been corrected to indicate the amount of enzyme on the basis of the original sample of ruminal ingesta.

[c] These values represent the sum of the activities in each pair of fractions (i.e., supernatant plus sediment) expressed as per cent of the activity in the starting material for that step.

cellulases of the ruminal flora, and was assumed in the present studies, in so far as particulate cellulases were concerned.

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# EFFECTS OF DIETARY DIETHYLSTILBESTROL ON SEVERAL BIOCHEMICAL FACTORS OF RUMEN INGESTA[1]

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**SUMMARY**

Two pairs of rumen-fistulated identical twin cows were used to determine some of the effects of low-level feeding of diethylstilbestrol (DES) on the rumen microflora. One twin of each pair was fed 10 mg. of DES per 1,000 lb. of body weight daily. Rumen ingesta samples were collected and determinations were made on pH, surface tension, total free volatile fatty acid concentration, percentage acetic, propionic, and butyric acids, in vitro gas production, and in vitro cellulose digestion as indirect indications of rumen microorganism activity.

DES did not cause a marked change in any of these measurements. However, there was a trend toward decreased in vitro cellulose digestion in the miniature artificial rumina inoculated with rumen liquor from cows receiving DES in their ration. The addition of 20 μg. of pure DES in vitro had no effect on cellulose digestion in the artificial rumina that had been inoculated with rumen liquor from the control cows, but was slightly stimulatory to cellulose digestion when inoculated with liquor from the treated cows.

It is concluded that the oral administration of DES by the addition of "Stilbosol" to the feed in amounts to furnish 10 mg. per 1,000 lb. of body weight daily did not have a marked effect on the microbial activity in the rumen of the cows studied.

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The effects of diethylstilbestrol (DES) on the digestibility of nutrients in the rations of ruminants have been reported by many workers, but the effects on rumen contents have been less thoroughly investigated. Several groups of workers (1, 9, 14, 15) reported that the inclusion of DES in the ration of ruminants did not significantly affect digestibility. Story *et al.* (18) reported that the digestibility of dry matter of a ration fed to lambs was increased by feeding 2 to 4 mg. of DES daily. Increases in the coefficients of digestion of cellulose and protein in the rations of wether lambs fed 10 to 20 mg. of DES daily also have been reported (3). The object of this study was to determine whether low levels of DES fed daily to cattle would affect the rumen microflora as measured by biochemical factors.

## EXPERIMENTAL PROCEDURE

*Animals and rations.* Two pairs of identical twin cows were used in this study. Each animal had a permanent rumen fistula fitted with a plastic cannula and cap. The cows were fistulated during the latter half of pregnancy and calved without difficulty. Both pairs of twins (81-82 and 31-32) were in their third lactations and were not bred during the experiment. One animal of each pair

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was fed the experimental ration containing DES and its twin was fed the control ration. In Twin Pair 81-82, Cow No. 82 was fed the experimental ration, since this animal had received it in the two preceding lactations. In the second pair (31-32) Cow No. 31 was chosen at random to receive the experimental ration. The cows were milked twice a day, at 6 A.M. and 6 P.M.

*Feeding methods.* A modified paired-feeding technique was used throughout the experiment. The cows were fed chopped alfalfa hay at a rate so that there was a small daily weigh-back. Concentrates were fed at a rate of approximately 1 lb. for each 4.0 lb. of 4% fat-corrected milk produced daily during the first 2 wk. of lactation. The amount fed was the same for each twin within a pair. The rations were fed at milking time.

DES was furnished in Stilbosol,[3] a concentrate containing 1 g. of DES per per pound. Sufficient Stilbosol to furnish 5 mg. of DES per pound of concentrate mixture was used as a replacement for part of the concentrate mixture of the treated animal of each pair. The formulas for the concentrate mixtures (14% total protein) were as follows:

| Control concentrate mixture | | DES-containing mixture |
|---|---|---|
| | *(lb.)* | *(lb.)* |
| Ground corn | 400 | 400 |
| Ground oats | 200 | 200 |
| Soybean oil meal | 100 | 96.5 |
| Salt | 7 | 7 |
| Steamed bone meal | 7 | 7 |
| Stilbosol | ..... | 3.5 |

The quantity of feed offered and the quantity refused were carefully weighed and recorded. In general, the ration was consumed in 2 hr. Water was available at all times in automatic watering cups.

*Biochemical methods.* Five 12-hr. trials were used for each pair of twins. Two of these trials served as a preliminary period for each pair before the start of DES feeding. Rumen samples were taken four times during each trial, at 5 and 10 A.M., and 2 and 4 P.M.

The water in the automatic drinking cups was turned off 1 hr. before each sampling. The 5 A.M. and 2 P.M. samples were taken from the rumen via the fistula, after the rumen ingesta had been mixed by hand for approximately 3–4 min. The 10 A.M. and 4 P.M. samples were taken after the entire rumen contents had been removed, weighed, and thoroughly mixed in a large tub. The emptying, weighing, sampling, and refilling of each rumen was done as rapidly as possible; usuallly, less than 30 min. was required. During the first four trials for each pair, pH and surface tension were determined on the 5 A.M. and 2 P.M. samples; whereas, during the last trial, these determinations plus those for volatile fatty acids (VFA) were made. The 10 A.M. samples were checked for pH, surface tension, VFA content, and total residual gas production from a 200-g.

[3] Supplied by Eli Lilly and Company, Indianapolis, Indiana.

sample. The 4 P.M. samples were checked for pH, surface tension, and VFA content. All samples were transported from the barn to the laboratory in glass screw-cap bottles. The samples were protected to prevent temperature change.

*pH.* A Beckman potentiometer was used for the determination of pH of the rumen samples. The pH readings were made on the rumen samples as soon as they were brought to the laboratory.

*Surface tension.* Rumen liquor, resulting from the squeezing, by hand, of rumen ingesta, was brought to the laboratory and allowed to stratify while being brought to 27° C. in a water bath. After a period of 30 to 45 min., the sample had stratified to such an extent that the straw-colored, clear layer could be strained through cheese cloth. The resulting liquor was used for surface tension determinations, using a du Nouy tensiometer. The average of three readings was recorded.

*Free volatile fatty acids (VFA).* Samples of liquor squeezed from the ingesta by hand were rapidly cooled, frozen, and held at approximately −23° C. until thawed for VFA determinations. The samples were thawed rapidly, but in a manner permitting the sample temperature to remain fairly cool.

Acetic, propionic, and butyric acids were separated by direct chromatography of rumen liquor on silica gel in a manner similar to that used by Mueller *et al.* (16).

The thawed rumen sample was mixed thoroughly and approximately 2 ml. clarified by centrifuging (2,000 G). One milliliter of the clarified sample was acidified with 1 ml. of 0.5 *N* sulfuric acid. One milliliter of the resulting acidified sample was added to 2 g. of washed and dried silicic acid, thoroughly mixed, and added to the top of the column after chloroform had been added to form a slurry.

Results of preliminary work indicated that the acid fraction reported as butyric contained traces of valeric acid. Other workers (8), using a similar technique, reported that the butyric acid fraction contained traces of higher acids; therefore, it was assumed that the fraction reported as butyric in the present study contained traces of higher acids present in the rumen liquor.

To determine the total quantity of VFA in the rumen, composite samples of 400 to 600 g. of rumen ingesta were collected at the 10 A.M. and 4 P.M. samplings, accurately weighed to the nearest gram, and dried in a forced air oven at 79° C. for 24 hr. The percentage of liquid present in the sample was calculated and applied to the total weight of rumen ingesta, to arrive at the quantity of liquid in the rumen. The total quantity of VFA present in the rumen was then calculated.

*Gas production.* Two-hundred-gram samples of rumen ingesta were collected at the 10 A.M. sampling for determining the total amount of gas produced. Samples were weighed into quart jars, placed in a constant temperature (39 ± 1° C.) water bath, and connected by a rubber tube to a gas-collecting cylinder. The gas-collecting vessel consisted of a 450-ml. calibrated glass cylinder containing water.

During a preliminary trial, a saturated salt solution (13) was used in the cylinders instead of water, and $CO_2$ was used to sweep the air out of the fermentation flasks before they were stoppered and transported to the laboratory. Hungate *et al.* (12) reported that there was no apparent difference in the mi-

crobial activity between rumen ingesta that were gassed with $CO_2$ before transporting to the laboratory and ingesta that were not gassed.

Neither using the saturated solution nor gassing the sample with $CO_2$ appeared to change the amount of gas produced and, consequently, neither procedure was used after the preliminary trials.

During the last two trials (IV and V), a solution of DES was added to two of the four fermentation flasks containing ingesta from each animal to determine the effect of DES on gas production in vitro. Forty $\mu$g. of pure DES was added to the 200-g. sample of rumen ingesta in Trial IV, and 80 $\mu$g. was added in Trial V.

*Cellulose digestion (in vitro).* The miniature artificial rumen described by Hanold *et al.* (11) was used to determine the effect of DES on cellulose digestion in vitro.

Samples of rumen liquor were collected at 3 P.M. The rumen contents were stirred, as previously mentioned, and the rumen liquor was squeezed through four thicknesses of cheese cloth and transported to the laboratory with a minimum of heat loss. In each artificial rumen was placed 500 mg. of finely ground alfalfa as substrate and 10 ml. of strained rumen liquor as inoculum. The ground alfalfa hay contained 29% cellulose. One trial, using 24 miniature artificial rumina, was conducted. Six miniature rumina were inoculated with the liquor from each cow. Two of the six for each cow contained 10 $\mu$g. of DES, two contained 20 $\mu$g. of DES, and the remaining two contained only the substrate and inoculum. Pure DES was used for all in vitro studies.

The contents of the miniature rumina were incubated for 24 hr. in a constant temperature water bath at 39 $\pm$ 1° C. At the end of the fermentation period, microbial activity was stopped by the addition of ethanol. Cellulose determinations of the contents of each miniature rumen were made, employing the method of Crampton and Maynard (7). The difference in the amount of cellulose at the start and that remaining after fermentation was considered to be the amount digested. To determine the amount of cellulose at the start, 10 ml. of rumen liquor from each animal was added to a beaker containing 500 mg. of ground alfalfa hay. Fermentation was prevented by the addition of ethanol. Cellulose determinations were then made on these samples at the same time as those that had been digested.

## RESULTS

*pH.* The mean pH values of rumen ingesta measured in vitro of each of the four daily samplings during the preliminary (Trials I and II) and test periods (Trials III, IV, and V) are shown in Table 1. It is evident from the uniformity of the pH values between twins within a pair that the feeding of DES at low levels did not measurably affect the pH of the rumen ingesta. There was a change in the average pH between the preliminary and test periods; however, this difference was evident in both treated and control animals of both twin pairs.

In general, there was greater uniformity of pH readings between twins within any one trial than there was between the readings for the same cow among the various trials. Since the animals were fed and managed as nearly as possible

TABLE 1
Effect of DES on pH of rumen contents collected at four periods during the day from identical twin cows

| Trial No. | Cow No. | DES fed daily/ 1,000 lb. body wt. | Mean pH value of rumen ingesta | | | | Mean for Trials (1 and II) and (III, IV, and V) |
|---|---|---|---|---|---|---|---|
| | | | 5 A.M. | 10 A.M. | 2 P.M. | 4 P.M. | |
| | | *(mg.)* | | | | | |
| I and | 81 | 0 | 6.42±.10[a] | 6.08±.32 | 6.30±.07 | 6.52±.19 | 6.33 |
| II | 82 | 0 | 6.44±.10 | 6.11±.37 | 6.40±.20 | 6.60±.14 | 6.38 |
| III, IV, | 81 | 0 | 6.31±.33 | 5.92±.12 | 6.20±.10 | 6.47±.06 | 6.23 |
| and V | 82 | 10 | 6.37±.17 | 6.03±.07 | 6.33±.06 | 6.52±.06 | 6.31 |
| I and | 32 | 0 | 6.37±.00 | 6.07±.00 | 6.36±.07 | 6.50±.10 | 6.41 |
| II | 31 | 0 | 6.78±.00 | 6.03±.12 | 6.43±.00 | 6.56±.00 | 6.45 |
| III, IV, | 32 | 0 | 6.72±.00 | 6.17±.07 | 6.52±.04 | 6.62±.00 | 6.51 |
| and V | 31 | 10 | 6.77±.04 | 6.28±.04 | 6.58±.06 | 6.72±.04 | 6.59 |

[a] Standard error of the mean.

in the same manner throughout the experiment, the lack of uniformity between trials is evidence of the marked influence of environment.

*Surface tension.* In Table 2, a summary of the data collected on the surface tension of strained rumen liquor is presented.

There is not a uniform trend in the changes of the surface tension readings between the preliminary period and the treatment period for either pair of twins. The average of samples during the preliminary period and test period for Cow No. 81 of 53.7 and 54.7 dynes/cm, respectively, showed a trend toward slightly higher surface tension during the test periods. Rumen samples from Cow No. 82 showed the opposite trend in surface tension, decreasing from 56.1 dynes/cm in the preliminary period to 54.8 dynes/cm during the period of DES feeding. In the other pair of identical twins (31-32), the changes were: 54.9 to

TABLE 2
Effect of DES on average surface tension values for rumen liquor collected at four periods during the day from identical twins

| Time of sampling | Mean surface tension of strained rumen liquor | | | |
|---|---|---|---|---|
| | Preliminary period | Test period | Preliminary period | Test period |
| | | *(dynes/cm)* | | |
| | No DES | | DES[a] | |
| | 81 | | 82 | |
| 5 A.M. | 52.5 | 53.8 | 52.4 | 54.3 |
| 10 A.M. | 52.4 | 54.1 | 56.2 | 54.8 |
| 2 P.M. | 54.7 | 55.3 | 58.5 | 55.4 |
| 4 P.M. | 55.3 | 55.4 | 57.3 | 54.8 |
| Average | 53.7 | 54.7 | 56.1 | 54.8 |
| | 32 | | 31 | |
| 5 A.M. | 51.3 | 53.8 | 53.7 | 53.7 |
| 10 A.M. | 55.9 | 51.8 | 51.7 | 52.6 |
| 2 P.M. | 55.5 | 51.9 | 56.4 | 54.6 |
| 4 P.M. | 56.7 | 52.8 | 56.7 | 53.8 |
| Average | 54.9 | 52.6 | 54.6 | 53.7 |

[a] 10 mg. stilbestrol/1,000 lb. of body weight daily.

52.6 dynes/cm for treated Cow No. 31 and 54.6 to 53.7 dynes/cm for the control, Cow No. 32.

The surface tension of the rumen liquor was fairly constant for all of the animals studied. The mean and standard deviations in dynes/cm, of 20 determinations on liquor from each cow during the preliminary and test periods, were as follows: Cow No. 81—54.2 ± 2.6; No. 82—55.3 ± 2.3; No. 31—54.0 ± 2.1; and No. 32—53.5 ± 3.1. These values were in general agreement with those reported by others workers (2, 19), and the differences were not significant.

*Free volatile fatty acids (VFA).* The mean quantities of VFA found in the rumen ingesta during the five trials of this study are shown in Table 3.

TABLE 3

Effect of DES on average values for pH, total free volatile fatty acids (VFA), and the percentage of acetic, propionic, and butyric acids present in the rumen of identical twin cows

| Trial No. | Cow No. | Time | pH | VFA present: Total in rumen content | VFA present: m*M*/100 ml. rumen liquor | VFA present: Acetic | VFA present: Propionic | VFA present: Butyric |
|---|---|---|---|---|---|---|---|---|
| | | | | (*M*) | | (%) | | |
| I and II | 81 | 10 A.M. | 6.08 | 3.67 | 14.25 | 61.2 | 18.7 | 20.6 |
| | | 4 P.M. | 6.52 | 1.63 | 9.05 | 60.3 | 17.8 | 21.9 |
| | 82 | 10 A.M. | 6.11 | 2.66 | 15.41 | 61.6 | 18.3 | 19.7 |
| | | 4 P.M. | 6.60 | 1.44 | 8.58 | 64.2 | 15.3 | 20.6 |
| III,[a] IV, and V | 81 | 10 A.M. | 5.92 | 6.89 | 16.08 | 68.8 | 15.3 | 15.9 |
| | | 4 P.M. | 6.47 | 4.12 | 10.84 | 68.7 | 15.8 | 15.5 |
| | 82 | 10 A.M. | 6.03 | 5.83 | 14.79 | 68.5 | 16.2 | 15.4 |
| | | 4 P.M. | 6.52 | 3.52 | 9.40 | 64.6 | 16.9 | 18.9 |
| I and II | 32 | 10 A.M. | 6.07 | 4.53 | 12.98 | 68.5 | 16.4 | 15.2 |
| | | 4 P.M. | 6.50 | 2.23 | 7.70 | 69.4 | 14.5 | 15.9 |
| | 31 | 10 A.M. | 6.03 | 5.09 | 13.91 | 68.8 | 16.4 | 14.8 |
| | | 4 P.M. | 6.56 | 1.73 | 6.82 | 74.4 | 13.4 | 12.2 |
| III,[a] IV, and V | 32 | 10 A.M. | 6.17 | 4.85 | 10.14 | 67.8 | 16.5 | 15.8 |
| | | 4 P.M. | 6.62 | 3.33 | 9.00 | 67.1 | 15.6 | 17.4 |
| | 31 | 10 A.M. | 6.28 | 4.80 | 10.40 | 68.8 | 15.9 | 15.3 |
| | | 4 P.M. | 6.72 | 3.05 | 8.26 | 62.2 | 16.8 | 21.0 |

[a] Cows No. 82 and No. 31 received DES during Trials III, IV, and V (10 mg/1,000 lb. body wt/day).

During the preliminary period, the rumen ingesta from Cow No. 81 at the 10 A.M. samplings contained an average of 3.67 *M* of VFA, whereas the ingesta from Cow No. 82 contained only 2.66 *M;* however, this difference is not found in a comparison of the VFA per 100 ml. of rumen liquor. As shown in Table 3, these values were 14.25 and 15.41 m*M*/100 ml. of rumen liquor for Cows No. 81 and 82, respectively. The reason for this comparatively large difference in total moles of VFA was the difference in total weight of rumen contents of the twins during the first two trials. These observations point out the advantage

of knowing both the relative amounts of VFA in the rumen ingesta and the total weight of the ingesta. The weight of the rumen contents of these two cows was fairly close during Trials III, IV, and V, so valid comparisons can be made between Cows No. 81 and 82, using total moles of VFA and m*M*/100 ml. of rumen liquor. This comparison revealed that during the period of DES feeding, the control cow (81) had an average of 1.06 *M* (1.29 m*M*/100 ml.) more of VFA in the samples of ingesta studied at the 10 A.M. sampling than did the treated cow, No. 82.

The concentrations of VFA of Twin Pair 31-32 were similar throughout the experiment. The rumen content weights also were similar for these two cows, so that an accurate comparison between m mol of VFA/100 ml. of rumen liquor could be made. At the 10 A.M. sampling, Cow No. 31 had an average of 0.93 m*M*/100 ml. more of rumen liquor than did Cow No. 32. The difference in m mol of VFA of these two cows during the period of DES feeding was only 0.26. The average total VFA concentration in moles of acid during the period of DES feeding was 4.85 and 4.80 for 32 and 31, respectively.

The average percentages of each of the three acids separated (acetic, propionic, and butyric) were similar when compared for twins of a pair for the same period of time (Table 3).

The quantity of concentrate fed to Cows 81 and 82 was increased approximately 50% after Trial II. This increased concentrate feeding, plus an increased hay consumption, was apparently the reason for the higher total VFA concentration in the rumen contents of both cows, No. 81 and 82, during the last three trials.

*Gas production.* The volume of gases evolved from 200-g. samples of rumen ingesta fermented in vitro was recorded at the end of 1, 2, 3, 5, and 7 hr. The collected gases were released after each reading, and the average of the readings made on four separate gas-collection units for each animal were compared. There was good agreement among these readings of the four replicas.

The total gas evolved during the fermentation process of Trials I and II for Twin Pair No. 81-82 was greater than that from either animal during Trials III, IV, or V. The reason for this is unknown. However, as shown by the data of these studies, the fermentation processes can not be expected to remain constant from one period of time to another, even though the feeding regime remains unchanged.

Although the gas production is not the same for each of the treated animals as for its pair-mate, there is no uniform trend in the differences noted. The average total gas evolved during the 7-hr. fermentation period from the ingesta of Twin Pair 81-82, respectively, was 541 and 557 cc. during the preliminary period, and 384 and 373 cc. for the test period. The gas production results for Twin Pair No. 31 and 32 were more uniform throughout all of the trials. During the preliminary period, the rumen ingesta from Cow No. 32 produced more gas in both trials. The average total gas for Cows No. 31 and 32 during the preliminary period was 420 and 447 cc., respectively. During the period of treatment the ingesta from No. 31 gave more total gas during fermentation in all three

trials, with an average of 421 cc. as compared to 413 cc. for the control cow, No. 32.

The addition of DES to the fermenting ingesta from all four animals did not caues any marked change in the amount of gas evolved.

*Cellulose digestion (in vitro).* The results of the effect DES had on cellulose digestion are shown in Table 4.

TABLE 4
Effect of DES on in vitro cellulose digestion

| Contents of artificial rumina | | Cow providing inoculum | | | |
|---|---|---|---|---|---|
| | | 81 | 82[a] | 32 | 31[a] |
| | | Cellulose digested | | | |
| | | (%) | | | |
| Basal[b] (Control) | Av.[d] | 55.4±1.5[e] | 51.6±0.5 | 54.3±0.4 | 53.1±1.5 |
| Basal + 10 μg. DES[c] | Av. | 55.2±0.6 | 53.2±1.0 | 54.6±0.4 | 53.2±1.4 |
| Basal + 20 μg. DES | Av. | 55.4±0.2 | 56.2±1.1 | 54.7±0.3 | 54.6±0.9 |

[a] Cows No. 82 and 31 were receiving DES (10 mg/1,000 lb. body wt/day) in their ration at the time the rumen liquor was collected for this experiment.
[b] Basal indicates ground alfalfa hay.
[c] Pure DES added in vitro.
[d] Average of digestion in two artificial rumina.
[e] Standard error of mean.

Since Cows No. 82 and 31 were receiving DES in their daily ration, their rumen contents probably contained it; consequently, the control part of the experiment was also a test of the effect of feeding DES on in vitro cellulose digestion. When miniature artificial rumina were inoculated with rumen liquor from cows receiving DES in their ration, there was a slight decrease in the per cent of cellulose digested, as compared to the amount digested by inoculum from the two cows receiving no DES in their ration. The addition of 10 μg. of DES to the artificial rumina did not cause a marked change in the amount of cellulose digested. However, when 20 μg. of DES was added to the artificial rumina inoculated with rumen liquor from each cow, there was a small increase in the percentage of cellulose digested in vitro when the inoculum was obtained from treated cows. However, there was little or no change in the percentage of cellulose digested upon the addition of DES in vitro when the inoculum was obtained from untreated cows. The increase in cellulose digestion in the case of the treated cows was not of sufficient magnitude to result in an increase over the controls, but merely raised the quantity digested into the general range noted for the control cows.

Although the differences in the percentage of cellulose digested were small, the data indicate that in vitro cellulose digestion is affected by DES.

## DISCUSSION

That orally administered DES stimulates growth in beef cattle and sheep is a well established fact, and although there is disagreement among the workers in the field, it has been reported by several authors (5, 10) to stimulate milk production. The method by which these beneficial effects come about is not

established. It is generally thought that the nutritional benefit the bovine receives from the feeding of DES is the result of (1) the effect it has in the rumen or (2) its action upon the endocrine system. The latter of these two possibilities seems to be the more widely accepted. However, the reports of several authors (3, 18) indicate the possibility of a local ruminal effect of DES. Furthermore, Pfander *et al.* (17) concluded that orally administered DES had a beneficial effect on the utilization of nutrients through its action on the rumen microflora.

The results of the present study do not support the suggestion that the beneficial action of DES is due to its effect on rumen microorganisms.

As far as can be determined from the data collected, the feeding of low daily levels of DES to lactating dairy cows did not measurably influence (1) the pH of rumen ingesta, (2) the surface tension of strained rumen liquor, (3) the concentration of VFA in rumen ingesta, (4) the average percentage of each of the three major VFA, or (5) the total quantity of gas evolved during a 7-hr. in vitro fermentation of rumen ingesta. It seems likely that if DES had a marked effect (i.e., enough to cause a stimulation to growth or milk production by increased utilization of nutrients) upon the microflora of the rumen, some indication of it would have been observed during the course of the reported experiments. The only indication of influence exerted by DES on the microflora at the level fed was noted in the study of in vitro cellulose digestion.

The results indicate a trend toward lowered in vitro cellulose digestion by the rumen microorganisms from the two identical twin cows receiving DES in their ration, when compared to their respective pair-mates. This indication of decreased microbial activity was not noted in any of the other tests designed to establish relative microbial activity.

The report of Brooks *et al.* (3), which indicated that the addition of DES to the artificial rumen increases cellulose digestion, was not substantiated by the results of this study.

It seems from the results of these in vitro cellulose digestion studies that the decrease in cellulose digestion noted in the artificial rumina inoculated with rumen liquor from the treated cows is offset by the addition of 20 $\mu$g. of DES to the substrate. The implication of these observations is not fully understood. However, it might indicate that levels of DES higher than those used in this study are stimulatory to in vitro cellulose digestion.

From the data presented, it appeared that low level feeding of DES did not have a marked effect on rumen microbial activity. This would suggest that the beneficial response obtained in many studies in which low levels of DES were fed is a result of the action DES exerts after reaching the systemic circulation via the portal blood stream. However, since a lack of marked effect on some of the endocrine glands from animals fed low daily levels of DES has been reported (4) and increased nitrogen retention in DES-treated animals has been widely reported (16, 18, 20), it is possible that the liver is the site at which DES exerts its influence.

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# CHANGES IN TYPE RATINGS OF HOLSTEIN COWS DUE TO AGE, SEASON, STAGE OF LACTATION, CLASSIFIER, AND YEAR[1]

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**SUMMARY**

Ratings of 233 Holstein cows, classified a total of 2,272 times from 1945 to 1957 by official classifiers, were coded to maintain equal intervals (1.0) between ratings. Least-squares analysis using dummy variables measured the effects of age with ratings grouped into Lactations 1, 2, 3, 4, 5, 6+; season, spring or fall; stage of lactation, parturition to 60 days, 61-240 days, and 241 days to next parturition; and classifier, 1 through 13. Linear year effects also were measured. Analysis on a within-cow basis removed possible animal bias. Constants, calculated as partial regression coefficients, represented the additive effect of each environmental factor independent of all others.

On a within-cow basis, coded over-all ratings increased steadily with age, with a range of 0.92 between Lactation 1 and 6+; spring ratings were higher than fall by 0.07**; early- and late-lactation scores were higher (0.13* and 0.17**) than mid-lactation. The range among classifiers was 1.38. Trends among subratings were similar except that feet and legs, and fore udder, showed smaller age and greater classifier effects; in late lactation, dairy character decreased (0.17**) and body capacity increased (0.27**). The linear decrease in over-all ratings was 0.03** per year, with a range of 0 to −0.06** for the subratings.

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In establishing the true relationships between type and economically important characteristics in dairy cattle, it is necessary to establish a common basis for the comparison of animals with each other. Because of variation in many factors which may conceivably influence a single type value, such a value for a cow may not be an accurate indication of the cow's inherent characteristics. A single value adjusted for some of these outside influences is needed.

A previous investigation and review of the literature by the authors (7) revealed that several factors, environmental in character, systematically biased official type ratings. Though experts in their field, official classifiers were not able to avoid either transitory or consistent fluctuations in making their subjective evaluations of animals; often this variation has been described as human error. Real changes occurred in appearance of the animals with age and with stage of lactation. Classifications took place in different seasons and in different years. These five influences (classifier, age, stage of lactation, season, and year) could logically bias scores for over-all rating, and for the various subratings; they are hereafter referred to as environmental factors. Measurement and evaluation of these effects in an attempt to obtain more valid estimates of type seemed justified, and were the objectives of this investigation.

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Previous studies were hampered by difficulties encountered in the analysis of large amounts of data for a large number of major effects through use of desk calculators. The recent availability of an electronic computer (IBM 650) to this station has made possible more comprehensive investigations.

## EXPERIMENTAL PROCEDURE

Data covering 13 yr. (1945 to 1957) were available from official type classifications of the Holstein-Friesian herd owned by the New Jersey Agricultural Experiment Station. During this time, all adult females were classified twice annually. A more complete description of the population and classification schedule has been published previously (7).

A total of 278 cows were classified 2,708 times for the over-all rating and each of the eight subratings. As a part of a special project from 1945 to 1949, three classifiers rated all animals simultaneously and independently (6); from 1950 to 1957 only one classifier at a time did the scoring. Ratings were coded as follows: Excellent, rated as 6; Very Good, as 5; Good Plus, as 4; Good, as 3; Fair, as 2; and Poor as 1. Intervals between scores were thus kept equal.

Lactation numbers were used as a measure of age and ratings grouped into Lactations 1, 2, 3, 4, 5, and 6 or higher (6+). Cows with ratings in the 6+ group completed an average of 6.82 lactations. Ratings were also grouped according to season (spring or fall), and three periods according to stage of lactation: (1) from day of parturition to 60 days, (2) 61 days to 240 days, and (3) 241 days to subsequent parturition. During the 13-yr. period, 13 classifiers rated the animals. Several came only twice, but one came 11 times; the average was 4.18 times.

Data representing every available animal classification were assembled. Obviously abnormal ratings were then excluded, such as many of those closely preceding or following abortions, and many terminal classifications prior to disposal. Since rather complete milk production and health records were kept, individual attention was given to borderline cases. Also excluded were animals which had been classified only once. There remained 2,272 classifications of 233 cows for a mean number of ratings per cow of 9.75.

A scheme of analysis was developed (4) combining the dummy variable and least-squares procedures, as described by Hazel (2) and Lucas (3), with the standard analysis of multiple covariance.

The nine type characteristics were numbered $1 \ldots h \ldots 9$; these included over-all rating, general appearance, dairy character, body capacity, mammary system, fore udder, rear udder, feet and legs, and rump. Environmental categories were numbered $1 \ldots i \ldots 6$. The categories, and the specific variables $1 \ldots j \ldots v$, were distributed in the following manner:

| | Category *(i)* | Variable *(j)* |
|---|---|---|
| 1. | Over-all | 1 |
| 2. | Lactation number | 1 to 6 |
| 3. | Season | 1 to 2 |
| 4. | Stage of lactation | 1 to 3 |
| 5. | Classifier | 1 to 13 |
| 6. | Year | 1 |

The animals were numbered 1 . . $k$ . . 233, and the observations within each animal 1 . . $l$ . . o. The linear model representing these effects, which were assumed to be additive, was as follows:

$$Y_{hijkl} = \mu_h + \sum\sum_{ij} b_{hij} X_{hij} + a_{hk} + e_{hijkl}$$

where $Y_{hijkl}$ = the type score for characteristic $h$, at the $l$th observation on the $k$th animal under conditions described by the $j$th variables in the $i$th categories,

$\mu_h$ = the base value for characteristic $h$ when all the $X_{hij}$ are 0,

$b_{hij}$ = the constant representing the effect on characteristic $h$ of the $j$th variable in the $i$th category,

$X_{hij}$ = the independent variable taking the value 1 or 0 for $ij = 11$ to 5 13 (dummy variables), or the coded value for year, a continuous variable, $(ij = 61)$ ; the same for all values $h = 1$ to 9,

$a_{hk}$ = the effect on characteristic $h$ of the $k$th animal, and

$e_{hijkl}$ = the error term including random error and any interactions among the main effects which may have existed, though none was assumed to have existed.

In the scheme of analysis used, one equation in each category based on dummy variables, except one, could not be considered independent. The simplest solution was to set one constant in all but one category equal to zero. The other constants in the category were then measured not as deviations from mean but as deviations from the variable which was set equal to zero. Calendar year was incorporated as a continuous variable and thus was not deleted; year was coded by subtracting 1900 from the actual year, e.g., 1948 − 1900 = 48. Selection of constants to set equal to zero may be quite arbitrary. Lactation 4 was selected as a point at which the dairy cow reaches maturity in size and production. Spring was selected over fall, and Period 2 over Periods 1 and 3. Classifier 3 was selected because he had classified the most times (11), with the most animal-classifications (539), and thus afforded the maximum number of within-animal and within-classification comparisons with other inspectors.

The term $\mu_h$ thus reflected the base value, or mean for type rating $h$ for Lactation 4 $(ij = 24)$, spring (31), Period 2 (42), and Classifier 3 (53). With Variable 61 a continuous variable, $\mu_h$ also reflected extrapolation back to 0, the coded value for year 1900.

The $a_{hk}$ term, representing animal effects, was taken into consideration by the analysis of multiple covariance approach, in which three matrices of sums of squares and sums of products were set up: total, among cow, and within cow. Since the objective in this study was to eliminate the effect of genetic differences among cows on the constants, they were calculated from the within-cow matrix. When effects are determined by this procedure, and if the assumption that no real interactions exist is correct, each effect can be considered to be independent of, or adjusted for, all other effects.

## RESULTS

Unadjusted means of the ratings grouped according to lactation number, season, stage of lactation, and classifier are presented (Table 1), along with the percentage of the total number of classifications in each category. With over-all ratings, and with most of the subratings, these mean scores increased with age; fall ratings were slightly lower than those in spring; marked changes occurred during the lactation; and classifier means varied considerably.

The least squares estimates of the effects of the five environmental factors (Table 2), calendar year being included as a continuous variable, were those obtained on a within-cow basis. The 1 and 5% levels of significance also have been presented.

It was apparent that animal effects very nearly had been randomly distributed. Their removal did not affect to any great extent the constants obtained. The total (not shown) and within-cow constants were very nearly alike. Furthermore, both sets of constants were similar to the simple arithmetic differences in the means, probably indicating a lack of any important interactions.

The statistical significance should be interpreted with caution. In the case of age, trends were probably more important than actual significance. Since Lactation 4 lay between 3 and 5, these lactations could have been an integral part of a trend without being significant, and such was usually the case. A somewhat comparable situation existed with stage of lactation, since the tendencies were usually for ratings to drop from Period 1 to 2 and rise again in 3. However, tests of significance of season effects were quite valid, because only one comparison (fall vs. spring) was made. Similarly, with the linear calendar-year term, the tests of significance validly indicated the presence of year effects. With classifier effects, observation of the mean values for Classifier 3 showed this person to be one of the stricter classifiers. Thus, the more lenient classifiers usually differed significantly. Had an inspector been selected whose ratings were close to the mean, his immediate neighbors very probably would not have differed significantly.

On the last line of Table 2 are the percentages of the within-cow sums of squares which could be attributed to the combined effects of the five environmental factors. These values ranged from 14 (fore udder) to 28% (feet and legs). As indicated in the mathematical model, the remaining within-cow variation consisted of that which was truly random and that which was due to interactions of any of the environmental factors with each other. No interactions between these variables and the animals themselves would be included, however.

The increase in ratings with age did not proceed in a linear manner, as is shown graphically for over-all rating in Figure 1. The upward trend observed from the simple means was more pronounced than that from plotting the within-cow constants, due to the animal effects on the former; most low-classifying animals were eliminated from the herd as young cows and thus reduced the means for the lower lactation numbers but not for the higher numbers. The constants were plotted by using the mean for Lactation 4 as the base. An asymptotic curve

TABLE 1

Means of type ratings falling into various environmental categories

| Environmental category | | Per cent of total | Type ratings | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Over-all | General appearance | Dairy character | Body capacity | Mammary system | Fore udder | Rear udder | Feet and legs | Rump |
| Lactation | 1 | 30 | 3.34 | 3.45 | 3.89 | 3.90 | 3.32 | 3.39 | 3.37 | 3.10 | 3.47 |
| Lactation | 2 | 24 | 3.57 | 3.67 | 4.21 | 4.06 | 3.48 | 3.36 | 3.59 | 3.18 | 3.79 |
| Lactation | 3 | 19 | 3.90 | 4.00 | 4.47 | 4.30 | 3.73 | 3.56 | 3.79 | 3.29 | 4.03 |
| Lactation | 4 | 11 | 4.04 | 4.08 | 4.65 | 4.56 | 3.72 | 3.56 | 3.76 | 3.18 | 4.06 |
| Lactation | 5 | 6 | 4.10 | 4.19 | 4.76 | 4.62 | 3.83 | 3.54 | 3.94 | 3.39 | 4.10 |
| Lactation | 6+ | 10 | 4.44 | 4.43 | 4.86 | 4.82 | 4.16 | 3.95 | 4.31 | 3.82 | 4.29 |
| Season | S | 50 | 3.77 | 3.86 | 4.38 | 4.28 | 3.64 | 3.49 | 3.75 | 3.30 | 3.82 |
| Season | F | 50 | 3.70 | 3.78 | 4.24 | 4.17 | 3.55 | 3.50 | 3.60 | 3.21 | 3.87 |
| Stage | 1 | 15 | 3.70 | 3.79 | 4.36 | 4.05 | 3.62 | 3.50 | 3.76 | 3.47 | 3.78 |
| Stage | 2 | 42 | 3.63 | 3.71 | 4.37 | 4.11 | 3.47 | 3.36 | 3.56 | 3.17 | 3.76 |
| Stage | 3 | 43 | 3.85 | 3.93 | 4.24 | 4.40 | 3.71 | 3.64 | 3.76 | 3.25 | 3.94 |
| Classifier | 1 | 6 | 3.82 | 3.95 | 4.21 | 4.43 | 3.58 | 3.58 | 3.45 | 2.93 | 3.86 |
| Classifier | 2 | 8 | 3.58 | 3.51 | 4.31 | 4.36 | 3.53 | 3.19 | 3.51 | 3.21 | 3.80 |
| Classifier | 3 | 24 | 3.55 | 3.68 | 4.07 | 4.02 | 3.36 | 3.29 | 3.39 | 2.69 | 3.61 |
| Classifier | 4 | 6 | 3.85 | 4.19 | 4.07 | 4.18 | 3.54 | 3.47 | 4.03 | 3.99 | 4.48 |
| Classifier | 5 | 7 | 3.99 | 4.00 | 4.33 | 4.11 | 4.14 | 4.04 | 4.09 | 3.69 | 3.86 |
| Classifier | 6 | 6 | 3.80 | 3.94 | 4.56 | 4.54 | 3.72 | 3.57 | 3.99 | 3.28 | 4.35 |
| Classifier | 7 | 3 | 4.20 | 4.22 | 4.36 | 4.51 | 3.95 | 3.87 | 3.91 | 3.84 | 4.13 |
| Classifier | 8 | 4 | 3.67 | 3.67 | 4.13 | 4.17 | 3.43 | 3.18 | 3.74 | 3.45 | 3.55 |
| Classifier | 9 | 15 | 3.74 | 3.84 | 4.81 | 4.16 | 3.78 | 3.62 | 3.73 | 3.26 | 3.73 |
| Classifier | 10 | 4 | 4.10 | 4.24 | 4.47 | 4.64 | 3.99 | 4.06 | 3.98 | 3.99 | 4.11 |
| Classifier | 11 | 5 | 3.70 | 3.72 | 4.25 | 4.25 | 3.52 | 3.44 | 3.64 | 3.39 | 3.79 |
| Classifier | 12 | 6 | 3.52 | 3.51 | 4.14 | 4.11 | 3.27 | 3.20 | 3.28 | 2.90 | 3.63 |
| Classifier | 13 | 6 | 3.55 | 3.54 | 4.22 | 4.26 | 3.49 | 3.42 | 3.65 | 3.44 | 3.41 |
| Average | | | 3.74 | 3.82 | 4.31 | 4.22 | 3.60 | 3.50 | 3.67 | 3.25 | 3.84 |

TABLE 2

Effects of five environmental factors measured on a within-cow basis and expressed as deviations from zero

| Environmental category | | Type ratings | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Over-all | General appearance | Dairy character | Body capacity | Mammary system | Fore udder | Rear udder | Feet and legs | Rump |
| Lactation | 1 | −0.51** | −0.43** | −0.65** | −0.38** | −0.30** | +0.03 | −0.37** | −0.06 | −0.54** |
| Lactation | 2 | −0.29** | −0.24** | −0.30** | −0.25** | −0.14** | −0.02 | −0.15** | −0.05 | −0.25** |
| Lactation | 3 | −0.11* | −0.04 | −0.13* | −0.12* | −0.00 | +0.03 | +0.02 | +0.09 | −0.01 |
| Lactation | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lactation | 5 | +0.10 | +0.12 | +0.16* | +0.11 | +0.11 | −0.04 | +0.15* | +0.17* | +0.05 |
| Lactation | 6+ | +0.41** | +0.34** | +0.17** | +0.22** | +0.38** | +0.26** | +0.46** | +0.45** | +0.20** |
| Season | S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Season | F | −0.07** | −0.09** | −0.07* | −0.16** | −0.06* | +0.03 | −0.14** | −0.03 | +0.02 |
| Stage | 1 | +0.13** | +0.11** | +0.04 | −0.02 | +0.24** | +0.21** | +0.31** | +0.34** | −0.06 |
| Stage | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Stage | 3 | +0.17** | +0.19** | −0.17** | +0.27** | +0.19** | +0.21** | +0.20** | +0.07* | +0.20** |
| Classifier | 1 | +0.19* | +0.20** | +0.10 | +0.34** | +0.09 | +0.14** | −0.03 | +0.12 | +0.30** |
| Classifier | 2 | +0.07 | −0.14* | +0.27** | +0.38** | +0.07 | +0.04 | +0.18** | −0.64** | +0.04 |
| Classifier | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Classifier | 4 | +0.37** | +0.57** | −0.00 | +0.16* | +0.37** | +0.54** | +0.74** | +1.46** | +0.84** |
| Classifier | 5 | +0.27** | +0.16** | +0.18** | −0.18** | +0.53** | +0.51** | +0.46** | +0.77** | +0.32** |
| Classifier | 6 | +0.27** | +0.27** | +0.48** | +0.53** | +0.48** | +0.58** | +0.64** | +0.71** | +0.58** |
| Classifier | 7 | +0.51** | +0.42** | +0.25** | +0.32** | +0.40** | +0.38** | +0.35** | +0.91** | +0.61** |
| Classifier | 8 | +0.12 | +0.01 | +0.06 | +0.10 | +0.02 | −0.17* | +0.33** | +0.70** | +0.10 |
| Classifier | 9 | +0.09* | +0.07 | +0.66** | +0.03 | +0.31** | +0.23** | +0.23** | +0.51** | +0.08 |
| Classifier | 10 | +0.25** | +0.29** | +0.25** | +0.23** | +0.24** | +0.40** | +0.21* | +0.97** | +0.49** |
| Classifier | 11 | +0.68** | +0.63** | +0.32** | +0.32** | +0.79** | +0.90** | +0.90** | +1.54** | +0.91** |
| Classifier | 12 | −0.68** | −0.75** | −0.21 | −0.19 | −0.90** | −1.05** | −0.98** | −1.31** | −0.82** |
| Classifier | 13 | −0.70** | −0.77** | −0.22* | −0.13 | −0.70** | −0.80** | −0.67** | −0.84** | −1.01** |
| Year | | −0.03** | −0.03** | −0.00 | +0.00 | −0.06** | −0.05** | −0.06** | −0.06** | −0.05** |
| Reduction (%) | | 22 | 20 | 22 | 17 | 18 | 14 | 21 | 28 | 23 |

** Significant at the 1% level.
* Significant at the 5% level.

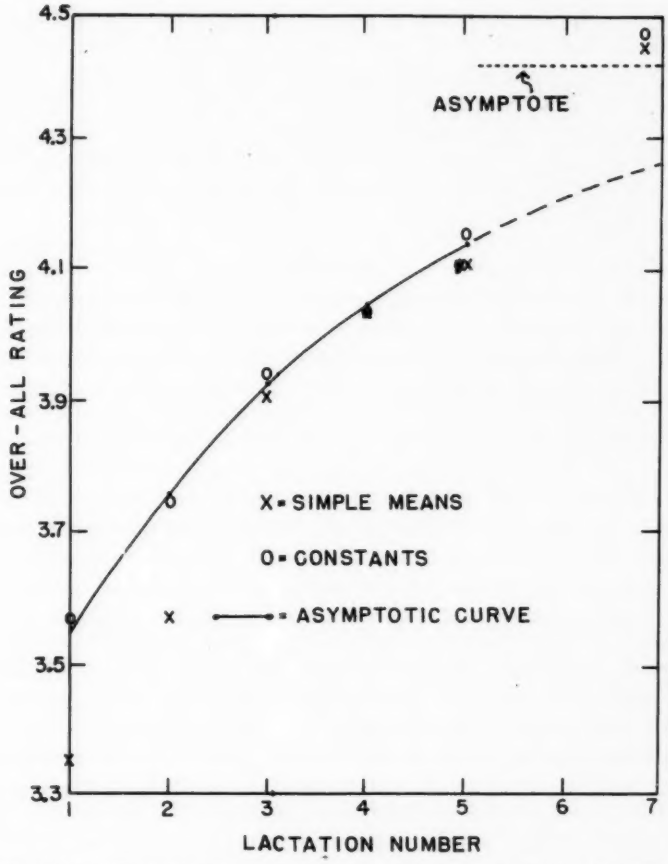


FIG. 1. An asymptotic curve representing the effects of age on over-all rating.

was calculated using the simple procedure described by Patterson (5); in this type of curve, the line approaches a maximum (asymptote) at an ever-decreasing rate. This might be expected in these data unless some other effect entered the picture. As possibilities, old age might have a tendency to reduce the rating, or greater leniency on the part of the classifier might tend to increase the rating more than otherwise would be expected. To evaluate the net effect of these possible influences, the asymptotic curve was fitted to the constants for Lactations 1 to 5, and extrapolated to the value for the Lactations 6+ data (mean 6.82 lactations) for comparison with the observed value.

The calculated curve fitted the plotted constants exceptionally well (Figure 1). The standard error of estimate of the within-cow constants was 0.0063 after fitting the curve. The closeness of fit also may be shown by the forecasting efficiency (1) of 97.4% and the correlation coefficient of .9997, though neither

of these measures is free of the influence of selection of the fixed independent variable.

With the very close fit of the curve to the data, an extrapolated value would be subject to relatively small error. For Lactation No. 6.82, the calculated expected value was $4.25 \pm 0.006$, which was markedly below the simple mean (4.44) and the comparable constant (4.45). These latter values also were slightly higher than the asymptote or theoretical maximum for an infinite number of lactations (4.41).

## DISCUSSION

When the confounding of the other environmental factors was removed from age, it was shown that a definite and statistically significant age effect remained. Where the range between grades was five score-card points, as with good to good plus, or good plus to very good, etc., over-all ratings of first-calf heifers differed from mature (6+) ratings by 4.6 points ($0.92 \times 5$). First-lactation animals could be expected to be raised almost one whole rating, on the average, if they remained in the herd long enough. Similar trends existed with all subratings except fore udder and feet and legs. Ratings for these two traits for animals in the sixth or later lactations were considerably higher than in Lactations 1 to 5, however.

The question of whether cows actually improved in type or whether they improved only in the eyes of the classifier must remain academic. It has been an admitted and probably desired policy of inspectors to be stricter with younger animals, and inspectors also have probably been more sympathetic toward the older, proven animals. It did not seem logical that feet and legs, or rumps, were actually improved by stresses of aging. It was also indicated, by the marked increase noted from Lactation 5 to 6+ in most type ratings, that the inspector's allowance for the very old cows was greater than it should have been. This was demonstrated by the differences between the observed and constant values and the expected value on the asymptotic curve (Figure 1).

Cows were rated higher after the winter feeding season than after the summer feeding season. The combined weight of more desirable mammary systems, body capacity, dairy character, and general appearance resulted in a higher (0.07**) over-all rating. The rather striking influence of season on rear udders was apparently great enough to affect mammary systems as a whole. It would seem that breeders striving for high average scores should classify in the spring, if their management and climatic conditions are similar to those of the population studied.

Over-all ratings were higher in late-lactation than in mid-lactation. Significantly lower dairy character ratings were more than offset by large increases in body capacity scores, along with smaller increases in all other subratings. Pregnancy and weight gains after the peak of lactation were probably responsible for increases in body capacity and general appearance; moreover, defects in mammary system were probably not as apparent in late-lactation as in mid-lactation. Shortly after freshening, animals were also consistently scored high. Though no significant changes occurred in dairy character, body capacity, and

rump, decreases in scores from early- to mid-lactation ranged from 0.11** (general appearance) to 0.34** (feet and legs) for the remaining ratings.

Removal of confounding environmental effects slightly accentuated differences among classifiers. Although the range among averages for over-all ratings was 0.68 (between Classifiers 7 and 12), the range for the calculated constants was 1.38 (for Classifiers 11 and 13). Differences were even more evident among the minor subratings, fore and rear udder, rump, and feet and legs.

During the last 5 yr. of the study, ratings were made by Classifiers 2, 3, 4, 6, and 11. Ranges among their means amounted to only 0.25 and among the constants only 0.68. With the advent of full-time professional classifiers, it is evident that some progress has been made in the establishment of a more uniform standard for classification.

Classifiers whose over-all ratings averaged higher or lower than Classifier 3 did not necessarily score the subratings likewise, although such was usually the case. The average deviation of over-all ratings of Classifier 2 was +0.07, but he was relatively strict on feet and legs (−0.64**) and easy (+0.38**) on body capacity.

Opposing the increase in scores with age were the decreases in the score by progressing calendar years. Negative in eight of nine ratings, and highly significantly so in seven of nine, these linear partial regression coefficients demonstrated a marked trend over the 13-yr. period. The underlying reasons for these effects were not clear, but it may be postulated that classification policies became stricter. Staff members who were associated with the herd for the whole period believed that the type of the herd had improved; however, the average score had not. Raising of standards by classifiers could have been the explanation. If true, of course, this by no means could be considered an undesirable trend. Difficult if not impossible to prove, similar trends were in evidence nowhere else in the literature.

With 22% of the within-cow variation among over-all ratings due to the five environmental factors, it would seem important to consider these factors in studies of type ratings and subratings. They should be considered to be a source of potential bias until proven otherwise. Gross differences among means should point out the major trends, if these trends exist in dairy cattle in general to the degree to which they existed in the Holstein population investigated.

If the decrease in the range among classifier means is indicative, it is possible that a more consistent standard has been reached by the classifiers in the recent years. It is probable, however, that the very old cows (6+) are still being given an over-allowance for age. Classifiers raised scores more in this period than expected from their previous systematic rate. Additional improvement would seem attainable in scoring dairy character and body capacity during the lactation. Since the stage of lactation of each animal is known to the classifier, he undoubtedly already adjusts his scores for these characteristics to some degree. If the true inherent type of the animal is best represented by all of its scores, however, the classifier is still being too strict on dairy character in late-lactation, and too lenient on body capacity during the same period.

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# EFFECT OF A SIMPLE HIGH-FIBER FEED ON DAIRY CALVES[1]

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**SUMMARY**

Thirteen pairs of calves including Jersey, Guernsey, and Holstein breeds were used to evaluate the effect of complexity and fiber content of concentrate mixtures for young calves. A simple high-fiber mixture containing 13.9% fiber was consumed as readily as was a complex low-fiber feed containing 6% fiber. No differences in tendency to consume hay were observed. All calves gained considerably more than the expected gain as computed by use of the Ragsdale standard (6), and no differences in rate of growth or general health were apparent.

Rumen functions develop considerably earlier in the life of the calf than previously was thought. Fermentation was observed to be well established in the stomach of calves 20 to 30 days old (4). Close observation has shown that rumination occurred in calves as young as 2 wk. (8). It also has been shown by the Ohio workers (2) that a high roughage ration is practical for young calves. Under conditions of this experiment, a complex calf meal was not necessary, but whole milk was fed through 7 wk. of age.

Concentrate feeds with some characteristics of mature ruminant feeds also appeared to be practical. Whitaker *et al.* (9) found that addition of alfalfa meal and/or corn cobs and shucks to bring the fiber content of calf meal to 13% did not affect its value as a feed for young calves. The original concentrate feed, however, was quite complex.

A very simple ration containing corn, oats, soybean oil meal, molasses, and minerals was reported by Murley *et al.* (5) as being fully as satisfactory as was a complex starter for young calves. This ration, however, was not particularly high in fiber.

Hollon *et al.* (3) observed that hydraulic process cottonseed meal had toxic effects on young calves; whereas, screw process cottonseed meal was well tolerated. Whitaker *et al.* (9) used cottonseed meal which had been degossypolized, and Murley *et al.* (5) used soybean oil meal. Neither of these meals had toxic effects. The protein source most readily available in the Southeast, solvent process cottonseed meal, has not received attention as a constituent of simple calf feeds.

If young calves can use a concentrate which is simple and also is high in fiber, only one ration should be needed for all ages of dairy animals. In the present study an ordinary high-fiber, simple cow herd concentrate mixture containing solvent process cottonseed meal was compared to a low-fiber complex meal as feed for young calves.

## EXPERIMENTAL PROCEDURE

Sixteen calves born during the fall of 1957 and spring of 1958, and ten born during the fall of 1958 and spring of 1959, were paired when four days of age. The pairs were balanced with respect to breed, sex, body weight, and height at

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withers. One member of each pair was offered a complex calf meal containing 300 lb. ground shelled corn, 250 lb. ground oats, 100 lb. wheat bran, 130 lb. extraction process cottonseed meal, 41% protein, 100 lb. nonfat dry milk solids, 8.5 lb. common salt, and 17 lb. steamed bone meal. This mixture contained 18% crude protein and 6% crude fiber as determined by standard methods (1).

Pair-mates received a concentrate mixture identical with the one used for the station milking herd. It contained 600 lb. ground snapped corn, 400 lb. citrus pulp, 200 lb. extraction-process cottonseed meal, 41% protein, 14 lb. common salt, and 28 lb. steamed bone meal. This mixture was 17.8% protein and 13.9% fiber.

All subjects were removed from their dams at birth. They were confined in individual pens which were bedded with wood shavings. Good-quality sun-cured Pangolagrass hay was offered free choice.

The milk ration offered in two equal portions daily was fed at the rate of 9% of body weight. Nipple pails were used during the first 3 wk. and thereafter milk was fed from open pails. Fresh colostrum, which was a mixture of the first four days' milk from Jersey, Guernsey, and Holstein cows, was fed during the first four days. A mixture of half colostrum and half skimmilk was fed between the ages of five and 21 days, inclusive. Thereafter, skimmilk was

TABLE 1

Effect of fiber contents of concentrates on growth and feed consumption by calves to 60 days of age

| Calf No. | Breed and sex | Body weight | | Height | | Feed consumed | |
|---|---|---|---|---|---|---|---|
| | | Initial | Gain | Initial | Gain | Concentrate | Hay |
| Control[a] | | *(lb.)* | | *(cm.)* | | *(lb.)* | |
| 1 | HF | 87 | 99 | 72 | 10 | 112 | 7 |
| 3 | HM | 102 | 92 | 77 | 9 | 60 | 28 |
| 5 | JM | 85 | 75 | 74 | 12 | 66 | 7 |
| 7 | GM | 81 | 63 | 79 | 8 | 55 | 10 |
| 9 | JM | 56 | 42 | 68 | 7 | 67 | 15 |
| 11 | GF | 78 | 62 | 75 | 10 | 52 | 6 |
| 13 | JM | 60 | 60 | 68 | 11 | 52 | 12 |
| 15 | JF | 53 | 49 | 64 | 10 | 46 | 13 |
| 17 | JF | 52 | 45 | 66 | 9 | 37 | 12 |
| 19 | GM | 79 | 58 | 75 | 8 | 56 | 13 |
| 21 | JF | 55 | 47 | 68 | 7 | 35 | 22 |
| 23 | JM | 60 | 37 | 69 | 7 | 46 | 13 |
| 25 | HF | 97 | 50 | 79 | 12 | 52 | 7 |
| Average | | 73 | 60 | 72 | 9 | 57 | 13 |
| High fiber[b] | | | | | | | |
| 2 | HF | 84 | 85 | 70 | 11 | 91 | 5 |
| 4 | HM | 102 | 95 | 81 | 9 | 55 | 32 |
| 6 | JM | 85 | 80 | 73 | 10 | 84 | 6 |
| 8 | GM | 81 | 53 | 75 | 11 | 74 | 11 |
| 10 | JM | 57 | 46 | 66 | 9 | 48 | 9 |
| 12 | GF | 78 | 50 | 74 | 7 | 39 | 5 |
| 14 | JM | 60 | 52 | 66 | 8 | 60 | 15 |
| 16 | JF | 55 | 57 | 66 | 9 | 74 | 14 |
| 18 | JF | 55 | 55 | 67 | 8 | 54 | 10 |
| 20 | GM | 75 | 65 | 74 | 10 | 88 | 4 |
| 22 | JF | 53 | 55 | 67 | 10 | 42 | 3 |
| 24 | JM | 60 | 50 | 69 | 8 | 64 | 10 |
| 26 | HF | 97 | 83 | 77 | 10 | 62 | 35 |
| Average | | 72 | 64 | 71 | 9 | 64 | 12 |

[a] The control ration contained 6% of fiber.
[b] The experimental ration contained 13.9% of fiber.

fed. The skimmilk was reconstituted at the rate of 1 lb. of air-dry solids per gallon. Vitamin A, 15,000 I.U. per head daily, was dispersed in the milk.

All concentrates and hay in the feeders were removed once daily, the consumption rate recorded, and a fresh offering supplied. Weekly records were maintained on body weights and heights at withers; observations were made three times daily to determine whether differences occurred in general appearance and health. The records were terminated when the calves reached 60 days of age, and all data were analyzed statistically, using the technique described by Snedecor (7) for paired data.

## RESULTS AND DISCUSSION

No scouring or other abnormalities were observed, all calves appeared vigorous, and no differences in general appearance and health were discernible. Two calves in the high-fiber group consistently selected the fine particles, leaving, for the most part, corn-cob particles in the feed box.

Calves in both groups consumed both concentrates and hay readily (Table 1). Growth was above normal (6) in all calves, and type of concentrate feed did not have any effect which could be considered significant. These data confirm the results of Whitaker *et al.* (9) and Murley *et al.* (5). They show further that the concentrate feed for young calves may be both simple and high in fiber.

The effect of solvent process cottonseed meal was not planned as a part of this experiment. It was included in both rations and, though no negative control was maintained for comparison, it appeared to have no ill effect. In so far as complexity and fiber content are concerned, there appears to be no reason to supply special concentrate mixtures for young calves under the conditions of this experiment.

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# TECHNICAL NOTES

## TEMPERATURE INCREASE DUE TO HOMOGENIZATION

The approximate temperature increase in °F. in a product due to homogenization may be estimated by dividing the homogenizing pressure (p.s.i.) by 300. More accurate estimates are obtained by dividing the homogenizing pressure by factors specific for a given product.

Many teachers and technologists refer to the temperature rise in the product caused by homogenization in terms of their casual observations, without regard to the variation with pressure.

It is readily seen that the temperature rise must be the result of energy input dissipated as heat. This heat is the equivalent of the hydraulic horsepower input. An equation for temperature rise can be derived as follows:

B.t.u.'s gained by product/hour = weight of product/hour × temperature change (°F.) × specific heat

$$\text{B.t.u.'s} = W\,\Delta t\,s \qquad (1)$$

B.t.u.'s expended by homogenizer = B.t.u. equivalent to horsepower hour × hydraulic horsepower = 2,545 h.p. (2)

$$\text{Hydraulic horsepower} = \frac{\text{weight of product/hour} \times \text{feet head}}{33{,}000 \text{ ft. lb/min} \times 60 \text{ min.}} = \frac{WH}{1{,}980{,}000} \qquad (3)$$

Equation (1) must equal (2)

$$W\,\Delta t\,s = 2{,}545 \text{ h.p.}$$

Now substitute (3) for h.p.

$$W\,\Delta t\,s = \frac{2{,}545\,WH}{1{,}980{,}000} = \frac{WH}{778}$$

Solve for $\Delta t$:

$$\Delta t = \frac{WH}{778\,Ws} = \frac{H}{778\,s} \qquad (4)$$

To use the homogenizer gauge pressure ($p$) directly, convert feet of head ($H$) to pounds per square inch (p.s.i.) pressure ($p$):

$H = \frac{p}{d}$, where $d$ = weight of product contained in a 1-in.-square column, 1 ft. high.

$= \frac{p}{.425}$ for water (at 150° F. homogenizing temperature).

Then $\Delta t = \frac{H}{778\,s} = \frac{p}{778\,s\,.425}$

and since specific heat, s = 1 for water

$$\Delta t = \frac{p}{331} \text{ for water}$$

Table 1 gives dividers for other products where more accurate estimates are desired.

The equation shows that the flow rate and type of homogenizer have no effect on the temperature rise.

TABLE 1

Dividers for converting pressure (p.s.i.) to ° F. temperature rise

| Product | Density factor (150° F.) $d$ | Specific heat[a] $s$ | Divider $778 \cdot ds$ |
|---|---|---|---|
| Water | .425 | 1.00 | 331 |
| Milk | .440 | .93 | 318 |
| 12% Cream | .435 | .90 | 305 |
| 20% Cream | .432 | .88 | 296 |
| Evaporated milk | .454 | .92 | 325 |
| Ice cream mix | .464 | .80 | 289 |

[a] Values taken from tables and/or extrapolated from curves given in Chapter 1, Arthur W. Farrall, *Dairy Engineering*, 2nd ed. John Wiley & Sons, Inc., New York. 1953.

TABLE 2

Comparison of temperature rise observed on actual trials with computed value-water

| Homogenizing pressure | Temperature rise, °F. 400 gal/hr[a] | 4,000 gal/hr[a] | Computed |
|---|---|---|---|
| *(p.s.i.)* | | | |
| 600 | .... | 2.0 | 1.8 |
| 800 | 2.5 | 2.5 | 2.4 |
| 1,000 | 3.4 | 3.2 | 3.0 |
| 1,200 | 4.0 | 3.6 | 3.6 |
| 1,400 | 4.5 | 4.2 | 4.2 |
| 1,600 | 5.0 | 5.0 | 4.9 |
| 1,800 | 5.6 | 5.3 | 5.4 |
| 2,000 | 6.3 | 6.3 | 6.1 |
| 2,200 | .... | 6.8 | 6.7 |

[a] Average of three to five measurements.

To check the actual temperature rise at various homogenizer pressures against the approximate computed values, a 400–g.p.h. homogenizer and a 4,000–g.p.h. homogenizer were set up in the laboratory, with copper-constantan themocouples attached at the intake and discharge connections. Water was pumped through the homogenizers and subjected to pressures ranging from 800 to 2,200 p.s.i. The temperatures of the water at the intake and at the discharge were measured with the thermocouples and a 12-pt. Brown Electronik potentiometer. The results given in Table 2 show very close agreement with the computed values.

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# SOME CHARACTERISTICS OF THE GLYCO-MACROPEPTIDE OF CASEIN—A PRODUCT OF THE PRIMARY RENNIN ACTION[1]

The primary action of rennin on casein and the concurrent release of nonproteolytic NPN have been the subject of recent investigations. Recently, Wake (5) showed that κ-casein, the calcium-insensitive protective colloid of the α-casein complex, was the substrate for the primary action of rennin. Alais (1) isolated two groups of peptides from the NPN fraction. One group, soluble in 2% TCA, was found to be electrophoretically heterogeneous and contained a major component which constituted 90% of the total 12% TCA-soluble constituents. The electrophoretic mobility of this component was reported at 7 to 8 $cm.^2 v.^{-1} sec.^{-1} \times 10^{-5}$ in veronal buffer at pH 8.6. Nitschmann *et al.* (4) found this component, ranging in molecular weight between 6,000 and 8,000, to be rich in carbohydrate and, consequently, the term glyco-macropeptide was used to describe the peptide.

The objective of our studies has been to elucidate the composition and physical characteristics of this electrophoretically homogeneous glyco-macropeptide.

Casein was prepared by isoelectric precipitation from fresh skimmilk, reprecipitated twice from alkaline solution, and subsequently washed. The wet casein was made up to 3% concentration in dilute NaOH, adjusted to pH 7.0 with 10% HCl, and dialyzed against distilled water for 12 hr. at 4° C. Crystalline rennin (Sigma Chemical Co.) was added to the sodium caseinate sol and the reaction allowed to proceed for approximately 1 hr. at 25° C. At this point, the ascending electrophoretic pattern showed a split in the α-casein peak.

The enzyme-treated sol was adjusted to pH 4.6 to precipitate the casein. The clear supernatant was dialyzed to remove the HCl and concentrated by pervaporation. A 20% solution of freshly prepared TCA was added slowly to the concentrated solution with constant stirring until a final concentration of 12% TCA was reached. The precipitated peptides were removed by centrifugation ($\simeq 1{,}000 \times G$ for 30 min.). The clear supernatant was adjusted to pH 7 with NaOH solution and dialyzed to remove TCA. The TCA extraction step was repeated two times before lyophilizing the glyco-macropeptide solution.

The electrophoretic characteristics of the macropeptide were observed in the following buffers: acetate, pH 4.2 and 4.7; phosphate, pH 5.9 and 7.4; veronal, pH 8.6 (pH values at 23° C., $\Gamma/2 = 0.1$). All of the solutions for electrophoretic analysis contained 1.5% macropeptide. The electrophoretic patterns are shown in Figure 1. The peptide appears to dissociate at low values of pH as indicated by the lack of homogeneity and flatness of the migrating boundary. The peptide migrates as a single, homogeneous component in buffers of higher pH values. Mobility values are presented in Table 1. An extrapolation of the descending mobility values to zero mobility gives an $I_e$ (pH of no electrophoretic migration) value of approximately pH 2.8.

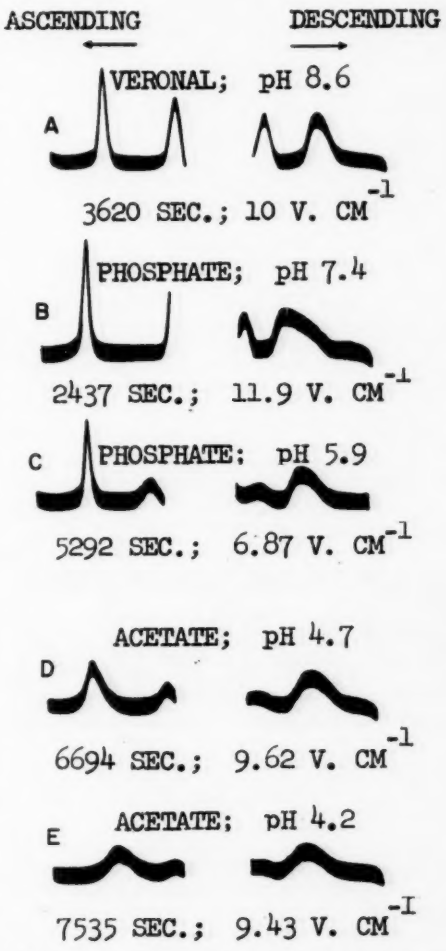


FIG. 1. The electrophoretic patterns of the glyco-macropeptide in various buffers. Concentration, 1.5%.

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TABLE 1
Electrophoretic mobility of the glyco-macropeptide in various buffers

| Buffer[a] ($\Gamma/2 = 0.1$) | | Electrophoretic mobility[b] ($\mu = \times 10^{-5}$ cm.$^2$ volt$^{-1}$ sec.$^{-1}$) Ascending pattern | Descending pattern |
|---|---|---|---|
| Veronal pH | 8.6 | 6.18 | 4.62 |
| Phosphate pH | 7.4 | 9.19 | 5.46 |
| Phosphate pH | 5.9 | 5.97 | 4.75 |
| Acetate pH | 4.7 | 3.46 | 2.64 |
| Acetate pH | 4.2 | 2.54 | 2.09 |

[a] pH measured at 23° C.
[b] Measured from initial boundary.

Sedimentation analyses were performed at 28° C. in a Spinco Model E Ultracentrifuge, with 1, 2, and 3% solutions of glyco-macropeptide in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$. The observed sedimentation constants were corrected in the usual manner and plotted. The $S_{20,w}$ values were extrapolated to zero concentration ($S^{c=0}_{20,w}$). This value was used in the calculation of the apparent molecular weight of the macropeptide. The sedimentation diagrams for the 3% solution and the plotted $S_{20,w}$ values for the 1, 2, and 3% solutions are shown in Figures 2 and 3, respectively.

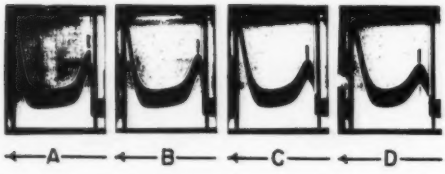


FIG. 2. The ultracentrifugal patterns of the glyco-macropeptide in the Spinco Model E Ultracentrifuge at 3% concentration in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$.

The diffusion characteristic was observed at 20° C. in a Perkin-Elmer 38-A Electrophoresis Apparatus equipped with a cylindrical lens. The quantities $A^2/4\pi H^2$, where $A$ and $H$ are the area and height of the diffusion peak, were plotted as a function of $t$, the measured elapsed time. The slope of this plot represents the diffusion constant ($7.5 \times 10^{-7}$). A partial specific volume ($V$) value of 0.68 was determined by means of the pycnometric method. Values determined for 1 and 2% solutions of macropeptide in veronal buffer at 20° C. were identical. Where required, relative viscosity values were determined by means of an Oswald viscometer. Density and viscosity values for water were taken from standard tables. The weight average molecular weight of the glyco-macropeptide, calculated from the sedimentation-diffusion method, was 15,263.

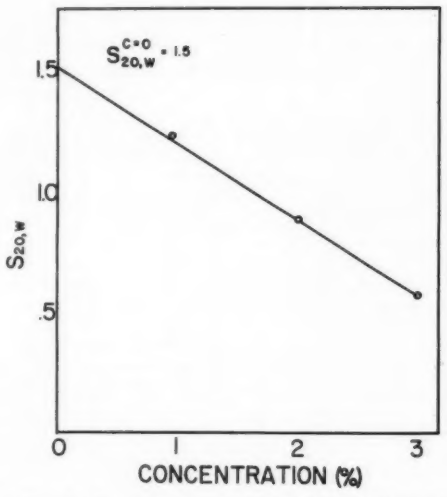


FIG. 3. $S_{20,w}$ plots for 1, 2, and 3% solutions of the glyco-macropeptide ($S^{c=0}_{20,w} = 1.5$).

Analyses for the various constituents of the glyco-macropeptide are listed in Table 2 and are compared with similar data reported previously by Nitschmann *et al.* (4) and Alais (1). Nitrogen, phosphorus, and neuraminic acid values were in good agreement. However, values for hexose and, to a lesser extent, glucosamine were lower in our glyco-macropeptide preparation. Nitschmann analyzed for the carbohydrate components by separation on chromatographic columns, whereas the techniques employed in this study are according to the scheme as outlined by Glick (2), which is capable of measuring individual protein-bound carbohydrates within a mixture. All of the standards were chemically pure, except for neuraminic acid which was obtained by using a standard sample of orosomucoid from hu-

TABLE 2
Chemical analyses of the glyco-macropeptide

| Analysis | Fraction per cent: Reported values | Values from this study |
|---|---|---|
| Nitrogen | 11.4[a] | 11.7 |
| Ash | .... | 6.0 |
| Phosphorus | 0.57[b] | 0.63 |
| Carbohydrate | | |
| Hexose (galactose) | 15.2[a,c] | 5.1 |
| Glucosamine | 4.3[a] | 2.3 |
| Neuraminic Acid | 11.4[a] | 11.3 |
| Total carbohydrate | 30.9 | 18.7 |

[a] Hs. Nitschmann *et al.* (4).
[b] C. Alais (1).
[c] Recently, Nitschmann (3) obtained a hexose value of 5.9% for a more homogeneous preparation of the glyco-macropeptide.

man serum as a secondary standard (11.2% neuraminic acid).

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## FORMATION AND REMOVAL OF AN IRIDESCENT DISCOLORATION IN CLEANED-IN-PLACE PIPELINES

In the course of some studies on in-place cleaning, Kaufmann *et al.* (2) reported the appearance of an iridescent discoloration on the interior of 18-8 stainless steel pipes after cleaning with an alkaline cleaning compound containing a nonionic wetting agent. Since this type of film has not been reported previously in the literature, this work was undertaken to determine some conditions of operation which would (a) cause the discoloration and (b) remove the discoloration.

*Film build-up.* The permanent CIP line used in this study consisted essentially of four sections of stainless steel pipe, each about 10 ft. long. Two sections were 500-grit finish, one was 300, and one was 180-grit finish. During the period of film deposition, the line was used for hot milk.

Immediately after use, the line was flushed with tap water at 90° F. and washed, using nonionic alkaline detergent "D" at the minimum recommended level. After flushing with tap water at 90° F. to remove the detergent solution, the line was chlorinated for 1-min., using 200 p.p.m. of chlorine. During soil build-up, circulation cleaning was carried out at 120° F. for 5 min. at a velocity of 4.8 ft. per second. Alkaline cleaner "D" contained polyphosphates, metasilicate, soda ash, and 2% nonionic wetting agent. The water hardness was not determined per se. Since the same supply was used in all studies, this factor remained a constant. Soiling with hot milk and cleaning as described was carried out for a period of 80 days. No acid cleaner was used during this period.

*Film removal.* Since film deposition occurred when cleaning was carried out at a low temperature and with minimum recirculation time, the first experimental cleaning trial was undertaken to study the effect of temperature on film removal. In the first trial, the temperature of detergent solution "D" was raised to 145° F. and recirculation carried on for 15 min.; the cleaning cycle was repeated 50 times. In the second trial, the temperature was raised to 160° F. and recirculation time increased to 30 min., since previous studies (2) indicated that a temperature of 160° F. for 120 min. removed the film. The results obtained in Trials I and II are given in Table 1.

In subsequent experimental cleaning trials, the CIP system was dismantled, to provide four individual pipe sections for study. Each section was cleaned as a separate unit on a CIP basis. This provided constant temperature control of the detergent solutions and constant recirculation velocity in each trial. With these factors held constant, it was possible to study the efficiency of various commercial detergents in removing iridescent film which had been permitted to build up. Each trial was carried out on the same section of pipe. When a procedure was found which removed the film, another soiled section of pipe was used to repeat the study, to eliminate interaction effects such as softening of the film by one process and removal by another.

In Trial III, anionic alkali No. 1 was used at the minimum level recommended by the manufacturer. This formulation differed essentially from the nonionic cleaner "D" used to produce the soil, in that it contained 8% anionic wetting agent and 6% less metasilicate. This solution was recirculated for 30 min. at 160° F. in Trial III. An alkaline bottle-washing compound was tested in Trial IV; this material also contained 8% anionic wetting agent, with a high level of alkalinity; it was recirculated for 30 min. at a temperature of

TABLE 1

Effect of various commercial cleaning compounds on the removal of iridescent discoloration in stainless steel pipes

| Triat No. | CIP cleaning procedure | | | Description of pipe interior following treatment |
|---|---|---|---|---|
| | Temp. | Time | Detergent used | |
| | (°F.) | (min.) | | |
| I[a] | 145 | 15 | Nonionic alkali D | Rainbow discoloration intense. Some black layer but no fatty deposits |
| II | 160 | 30 | Nonionic alkali D | Rainbow discoloration intense. No black spotting |
| III | 160 | 30 | Anionic alkali No. 1 | Rainbow discoloration |
| IV | 160 | 30 | Anionic alkali No. 2 | Rainbow discoloration |
| V | 160 | 20 | Organic acid | Rainbow discoloration |
| VI | 140 | 20 | Chlorinated alkali "A" | Some discoloration removed in less intense areas |
| VII | 140 | 20 | Chlorinated alkali "B" | No rainbow. Clean |

[a] This trial was repeated 50 times.

160° F. An organic acid detergent at a level of 1 qt. per 30 gal. of water was recirculated for 20 min. at 160° F. in Trial V and two chlorinated alkaline cleaning compounds were tested in Trials VI and VII. Chlorinated alkali "A" was employed at the recommended level of 15 oz. per gallon of solution; it contained sodium hypochlorite, sodium metasilicate, and sodium phosphate as active ingredients. Chlorinated alkali "B" was used at a level of 8 oz. per 30 gal. (one-half the recommended level); on a dry basis the formulation of "B" contained the same percentage of sodium hypochlorite as "A" but considerably less metasilicate and wetting agent. Recirculation cleaning was carried out in both of these trials at 140° F. for 20 min. The results of all trials are summarized in Table 1.

*Film deposition.* Nonionic alkali "D" showed some evidence of film formation after seven days of use. At this time some slight rainbow discoloration and light brownish blotches were visible. After 28 days of use, an iridescent film completely covered the interior of all pipes. The continued use of detergent "D" in the cleaning operation at 120° F. for 80 days resulted in the formation of a thick, black, fatty layer which could be loosened by mild rubbing; this deposit was scattered throughout all pipes. In the cleaner areas (no black layer) some rainbow discoloration was noted. It is apparent from this that detergent "D" at 120° F. permitted formation and build-up of the film under the conditions described. This is in marked contrast to the anionic alkali No. 1, which did not permit film formation under identical conditions of use (1). It is interesting to note that very, very little iridescent film was observed in the CIP system after 1 yr. when nonionic alkali "D" was used at 145° F. rather than at 120° F. The importance of temperature in the formation of this film is indicated. It is apparent from the data (Trial I) that raising the temperature to 145° F. after the iridescent film had formed failed to eliminate it. The fatty residue was removed at the higher temperature, but the black layer and intense iridescent film remained clearly visible even after 50 trials.

*Film removal.* Although temperature played a role in film deposition, increasing the temperature of nonionic detergent "D" from 120 to 160° F. did not remove the discolored areas (Table 1, Trials I and II). Under the latter condition, the dark black stain was removed, but the intense iridescent areas remained clearly visible on the dry surface. Although anionic alkali No. 1 did not permit the iridescent film to be formed at 120° F., it could not remove the film at 160° F. (Table 1, Trial III).

Anionic alkali No. 2, used in Trial IV, was not effective in removing the film, although the active alkalinity of this formulation was considerably higher than that of anionic alkali No. 1. Alkalinity per se apparently does not play a major role in the removal of this film.

The results obtained in Trial V indicate that the commercial organic acid cleaner did not remove the intense discoloration after recirculation at 160° F. for 20 min. The question as to whether weekly acid treatment as is recommended in routine plant operation would have removed the film formed in seven days can not be answered with assurance at this time. From the foregoing it is evident that the removal of this film, once it is present, is not a simple problem with certain alkaline or acid detergents. From this it is apparent that preventative rather than removal techniques are to be emphasized.

Both chlorinated alkalies used in Trials VI and VII exerted some action on the film, but the efficiency of removal varied considerably. Detergent "A," at the recommended level, removed some of the film in the lighter areas after circulation cleaning at 140° F. for 20 min.; detergent "B," however, at one-half the recommended level, removed the film com-

pletely under the same test conditions. These trials were both repeated using two pipes showing discoloration. Results similar to the above were obtained. The sodium hypochlorite concentration is the same in both detergent formulations. Since the quantity of detergent "B" used was about one-half that of "A," it follows that the actual level of chlorine varied in the detergent solutions. It is also important to note that efficient film removal was observed at the lower concentration of chlorine. It has been suggested that the low metasilicate content in detergent "B" might permit greater detergent action, owing to a more favorable pH for chlorine activity (1).

A cleaning procedure is described whereby an iridescent film was built up in a stainless steel pipeline by using a selected commercially available nonionic detergent at 120° F. Increasing the temperature of the nonionic and anionic cleaner to 160° F. did not remove the film after it had formed. A highly alkaline bottle-washing compound and an organic acid cleaner failed to remove the film. Chlorinated alkali detergents varied considerably in their ability to remove the iridescent film.

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ADDENDUM

Since the completion of this study, a strong iridescent discoloration was observed on the glassware used in our laboratories. Replacing the commercial detergent routinely used with chlorinated alkali "B" removed the rainbow film on all test tubes and Petri plates.

## PREPARATION OF A STANDARD FOR CITRIC ACID ANALYSIS

Considerable difficulty has been experienced with crystalline citric acid as a basis for preparation of a standard solution. Both the anhydrous and monohydrate forms of the acid are unstable at room temperature and humidity, with the result that the water content can vary between 0 and 2.5 *M/M*. Although the anhydrous acid is stable in a desiccator, it is difficult to obtain it by drying the monohydrate, because crystals of citric acid tend to melt at temperatures above 75° C. (4). Consequently, we have studied trisodium citrate as a standard material.

The composition of trisodium citrate dihydrate was verified by analyzing two lots (reagent grade) obtained from different manufacturers. The salt was of high purity and was probably obtained by recrystallization under controlled conditions of temperature and pressure (2). Sample A was taken from a freshly opened bottle, whereas the bottle containing Sample B had been opened for at least 1 yr. No preliminary drying was given either sample before analysis. Carbon was determined by dry combustion (1), water was estimated by heating to 150° C. overnight (4), and sodium was measured by flame photometry (5). The following results were obtained:

| | CARBON (Theor.: 24.50) | | WATER (Theor.: 12.25) | | SODIUM (Theor.: 23.46) | |
|---|---|---|---|---|---|---|
| | A | B | A | B | A | B |
| Found, % by wt.: | 24.28 | 24.36 | 12.36 | 12.19 | 22.70 | 22.95 |

All of these results fell within the experimental error of the various techniques.

Although this study indicated that trisodium citrate dihydrate was stable at room temperature and humidity, its stability was further studied by storing for three-day intervals under various extreme conditions. Exposure to dry 80° C. air caused a small loss in weight (0.041%). At room temperature, storage in the desiccator resulted in a negligible loss of weight (0.003%), whereas holding at 100% relative humidity caused a gain in weight of 3.65%. It is, therefore, apparent that trisodium citrate dihydrate is quite stable under normal laboratory conditions.

In a previous publication (3), a stock solution containing 50 mg. of anhydrous citric acid per milliliter was recommended, from which suitable standards could be prepared. On the basis of the above study of sodium citrate, the following preparation is now recommended: 76.4712 g. of trisodium citrate dihydrate (reagent grade) is made up to one liter with water. This solution is 0.260 *M* and, therefore, compares with one containing 50 mg. of anhydrous citric acid per milliliter. The solution is stored at 0° C. Fresh dilutions

of this stock solution are prepared each month and are also stored at 0° C.

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# THE METABOLISM OF SERINE-3-$C^{14}$ BY THE LACTATING SHEEP MAMMARY GLAND [1,2]

The contribution of serine to the metabolism of the mammary gland in capacities other than protein synthesis is unknown. Recent reports of Wood *et al.* (6, 7) qualitatively show that serine can be formed in the mammary gland from glucose and glycerol. The conversion most likely occurs by way of phosphoglycerate and phosphohydroxypyruvate (3). A reversal of this reaction sequence could lead to the formation of phosphoglyceraldehyde from serine. Since triose phosphate isomerase is active in the gland (7), it would be possible for serine to contribute to the formation of glycerol.

To study the metabolism of serine by the mammary gland, 90 $\mu$c. of serine-3-$C^{14}$ was placed, immediately after milking, into the gland cisterns of the udder of each of two lactating ewes. After 8 hr., the ewes were milked, sacrificed, and the glands removed for analysis. Various substances (Table 1) were isolated from the milk and mammary gland tissue by conventional means and assayed for radioactivity in a thin-window gas-flow G.M. counter. Both milk and mammary tissue were analyzed to eliminate the effect of differences in secretion rates of the various milk components on the distribution of $C^{14}$.

During the 8-hr. period between the addition of the labeled compound to the udder and the sacrifice of the animal, 94 and 87% of the labeled serine were utilized from the gland cisterns in Experiments A and B, respectively. Of the serine taken up, 16% was recovered from milk and glandular tissue in Experiment A, whereas 36% was recovered from these sources in Experiment B. Of this recovered $C^{14}$, 98% (Experiment A) and 99% (Experiment B) were located in the proteins of the milk and mammary gland. Table 2 shows the specific activity of the classical protein fractions in the milk from Experiment A.

The specific activity of casein and lactalbumin, in relation to the per cent serine in each (casein 6.3, lactalbumin 4.8), suggests that

TABLE 1

Distribution of recovered $C^{14}$ from serine-3-$C^{14}$ metabolized following administration to the lactating sheep udder[a]

| Metabolites isolated | Per cent of metabolized, recovered $C^{14}$ | |
|---|---|---|
| Milk | Exp. A | Exp. B |
| Triglyceride glycerol | Trace | Trace |
| Triglyceride fatty acids | 0.0 | 0.0 |
| Phospholipids | 0.2 | 0.1 |
| Proteins | 33.6 | 82.9 |
| Lactose | 0.0 | 0.2 |
| Mammary gland | | |
| Triglyceride glycerol | 0.4 | Trace |
| Triglyceride fatty acids | 0.0 | 0.0 |
| Phospholipids | 0.9 | 0.1 |
| Proteins | 64.7 | 16.7 |
| Glycogen | Trace | Trace |

[a] In Experiment A, 94% of the administered serine was metabolized, of which 16% was recovered in the substances isolated. In Experiment B, 87% of the serine was metabolized, of which 36% was recovered.

TABLE 2

Specific activity of the classical fractions of milk proteins following the arministration of serine-3-$C^{14}$ to the lactating sheep udder (Experiment A)

| Protein fraction [a] | Specific activity |
|---|---|
| | *(c.p.m/mg)* |
| Casein | 147 |
| Lactoglobulin | 49 |
| Lactalbumin | 103 |

[a] Nomenclature as suggested by Reference 4.

[1] Authorized for publication on June 5, 1959, as Paper No. 2368 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] Supported by the U. S. Public Health Service (H3632).

they arose from the same amino acid pool within the mammary gland. The formation of these proteins from the same amino acid pool previously has been suggested by the work of Askonas *et al.* (2). Another report by these workers (1) shows that at least a portion of the immune globulins passes directly from blood to milk. The dilution of synthesized globulin by the inclusion of preformed proteins from the blood could account for the low specific activity of lactoglobulin isolated from the milk in this experiment.

Although the percentage of recovered label in phospholipids is rather small, it indicates the incorporation of serine carbon into this lipid fraction. Since the serine was labeled in carbon 3, ethanolamine or choline formed from serine would still retain the labeled carbon in the molecule. The amount of these nitrogenous compounds present in the phospholipids of milk is very small when contrasted to the much greater quantities of serine in the milk proteins. This large difference in quantities present could explain the distribution of label between these two fractions.

The results indicate that serine did not undergo any major reactions in the mammary gland other than its incorporation into proteins and phospholipids. For serine to have been transformed as previously suggested it would first have to be phosphorylated. This would not appear to be a limiting step in the mammary gland, since the serine incorporated into casein is in the form of the phosphate ester.

Between 64 and 84% of the labeled serine removed from the gland cisterns could not be accounted for in the substances isolated from milk and glandular tissue. It is possible that the major portion of the serine was carried to other tissues of the body by blood traversing the mammary gland. In this regard, it is interesting to note the results of Rogers and Kleiber (5) with labeled acetate introduced in the same manner. These workers found that the majority of labeled acetate was utilized by the quarter into which it was placed. Thus, there may be a great difference in the rates of utilization of serine and acetate by the mammary gland. A slower entrance of serine into the metabolism of the gland could permit more to be carried from the udder without alteration. Another possibility, to account for the loss of $C^{14}$ label, would be the conversion of large quantities of serine to glycine. In this reaction, the labeled carbon would be removed from the serine molecule.

The method of placing labeled compounds directly into the gland cistern, as introduced by Rogers and Kleiber (5), appears to be an effective means of insuring, at least for water-soluble materials, that the desired metabolite is presented primarily to the mammary tissue without alteration by other tissues of the body.

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# OUR INDUSTRY TODAY

## BRUCELLOSIS ERADICATION MOVES AHEAD AT ACCELERATED PACE

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At the American Dairy Science Association's annual meeting in Indiana in 1940, the Dairy Cattle Health Committee of the Association was born in the Extension Section. Its first committee report was presented the following year at Vermont, before a joint session of the Extension and Production groups. From that time until the present, this committee, later becoming a joint committee of Extension and Production, has made its reports regularly. Brucellosis eradication in the United States from the start has been a prime objective. Now, in 1959, this once costly cattle disease is definitely and surely being brought under control—eradicated.

As of June 30, 1959, 19 states, the Virgin Islands, and Puerto Rico were designated as modified-certified brucellosis-free areas, a status meaning that over 99% of the cattle and over 95% of the herds are brucellosis-free. In addition to these areas, 829 counties in 29 other states also are certified. The most recent states to gain this enviable position were Nevada, Arizona, and Maryland. The first state was North Carolina in 1942, followed by New Hampshire in 1949.

The accompanying map of the United States shows the states and counties (in black) that were on the certified list, March 31. Education, legislation, testing, and calf-vaccination have enabled these areas to reach these goals.

### STATE PROGRAMS RECEIVE STUDY

Last summer and fall (1958), I had occasion, while on sabbatical leave from Cornell, to visit 18 western and southern states to study the livestock health problems and programs. Three of these states were certified: Washington, Utah, and New Mexico. Many of the others visited, while not certified, did have counties that had reached this status. Montana for instance had 50 of its 56 counties on the list. In Arizona, all but Maricopa County had reached this goal.

California, with only one county then certified (Del Norte in the north), was steadily pushing southward with its cattle-testing program. It was anticipated that by the end of 1959 about 36 counties would be certified, and that by December 31, 1960, the entire state would attain this coveted recognition. Texas

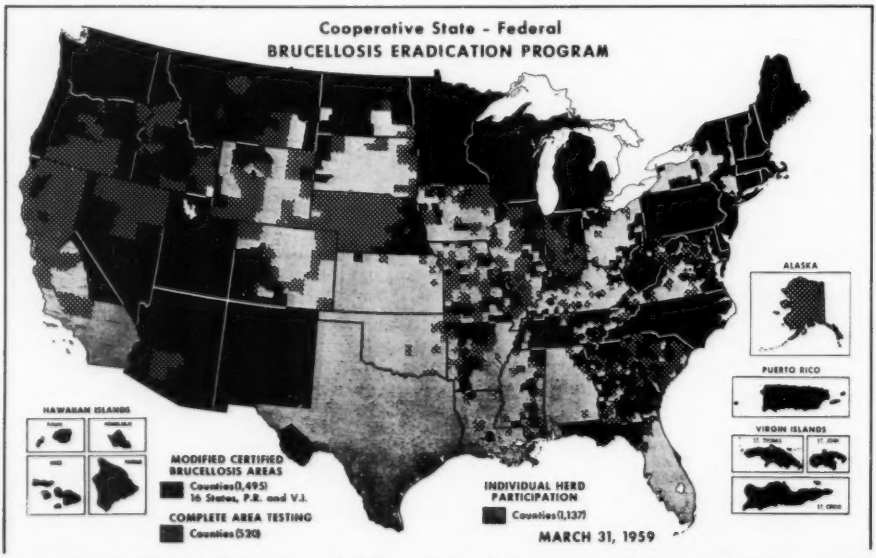


Fig. 1. United States map. Nineteen states have been approved as modified-certified brucellosis-free areas. Arizona, Maryland, and Nevada were recently added to the list.

has been slow to embark upon an official plan for brucellosis eradication. It is now moving forward with the support of the Texas Animal Health Council, and the Extension Service is actively promoting educational programs to stimulate eradication interest. Three counties in the southwestern part of the state are certified. However, since Texas is a big state of 254 counties, 4 yr. may elapse before state certification can be achieved.

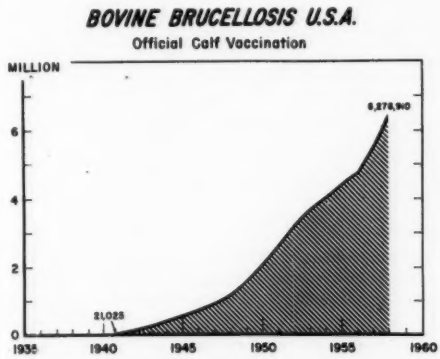


FIG. 2. Calf vaccinations. Calf vaccinations with Strain 19 vaccine have increased steadily since officially approved in 1940.

In all of the states visited upon this extended survey, brucellosis eradication and state certification were major projects and goals. The chief factors limiting more rapid progress appeared to be a lack of funds and insufficient numbers of veterinarians to keep the work progressing as rapidly as cattle owners and officials desire. However, to meet the personnel problems, local practicing veterinarians are being utilized upon a fee basis in many states, to supplement the services of state and federally employed veterinarians. This is speeding certification progress in these areas.

### MILK RING TEST, A BLOOD TEST SUPPLEMENT

The blood agglutination test has been the basic test for determining the presence or absence of brucellosis. With the advent and development of the milk ring test (ABR test) in recent years, another tool in the fight against this disease has emerged. Without its availability and utilization in the major dairy states now certified, Minnesota, Wisconsin, and Pennsylvania, it is doubtful if these states could have achieved their objectives at the time they did.

The milk ring test is a rapid, reliable, low-cost method for screening the brucellosis-suspicious herds from the negative herds. In comparing the accuracy of the milk ring test with the blood test, 3,615 herds and 73,155 cattle in two Wisconsin counties were tested. The ring test in this trial missed only one herd

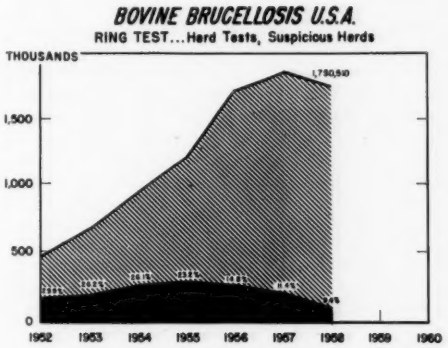


FIG. 3. Ring tests. Milk ring testing as a blood test supplement has made a major contribution to the elimination of brucellosis in dairy sections.

that contained a lone lactating reactor that proved to be a shedder of brucella organisms.

On the basis of cost, to locate each herd containing reactors in Calumet County, the expense was $1,014 by the blood test and $104 by the milk ring test method. In Oconto County the costs were: blood testing $536; ring testing $102.

Most states are using the milk ring test as routine procedure for dairy herds. When infected herds are suspected as a result of the ring test, the blood test is applied to locate the infected individuals. Reactors, if found, are tagged, branded, and removed to slaughter with or without indemnities, in accordance with the state plan of procedure. Thirty states pay some reactor indemnities.

The ring test is not accepted for area certification purposes in all states. This is true in states like Nebraska where many herds are mixed herds, beef and dairy cattle. The ring test used alone for these mixed herds does not give a true herd status. Therefore, under these conditions, only the results from blood tests are being accepted. As a rule, the dairy states utilize both blood tests and ring tests for area certification.

Another milk test known as the milk whey test has been developed in California. This new test is being tried out under field conditions in Marin and Sanoma counties, to determine its accuracy compared to the blood test in locating infected animals. These field trials are being watched with interest by federal and state sanitary officials, and by the research people who developed this test.

### SIX MILLION CALVES VACCINATED IN 1958

Calf vaccination with Strain 19 vaccine, after extensive field work in the late 1930's, was given official approval for use by the United States Department of Agriculture in December, 1940. Since that time, calf vaccina-

tion has become a major brucellosis control force in many states. Over 6,000,000 calves, 41% of those eligible, were officially vaccinated in 1958, according to a report of the United States Agricultural Research Service, Animal Disease Eradication Division. Each year, more calves receive these protective shots.

Last year, Wisconsin vaccinated 529,000 calves; Nebraska, 441,000; Montana, 308,000; California, 396,000; Missouri, 306,000; New York, 261,000; Iowa, 261,000; South Dakota, 273,000, and Kansas, 228,000. Since 1941, official records show that 42 million calves in the United States have received Strain 19 vaccine. So convinced are many buyers of replacement cattle of the protective value of calf vaccination that they refuse to consider the purchase of unvaccinated animals. In some areas, dairy nonvaccinates are selling at a discount price of $40 to $50 a head below the price of the vaccinates.

Vaccination has been a great boon to the brucellosis eradication effort in this country, especially in areas previously heavily infected as many intensive dairy areas once were, and in the range states where many ranchers have frowned upon blood testing as a method of brucellosis control. Fortunately, vaccination came along to fill this gap where test-and-slaughter procedures apparently failed to satisfy.

A problem arising from calf vaccination, so well has it met the needs in most range states, is that some people desire to discard the blood test and substitute vaccination as the major program method. Such a shift, in the opinion of brucellosis specialists, is a backward step from the present program aimed at complete eradication. Vaccination may largely control the economic effects of brucellosis, they say, but will not eradicate it. Most folks prefer not to live with this disease, so eventual removal of all reactors still remains state and federal long-time policy.

### UNIFORM METHODS AND RULES ADOPTED

For years, efforts have been put forth to formulate a brucellosis eradication program that meets the diverse livestock conditions prevailing in the various states and regions. The National Brucellosis Committee and the United States Livestock Sanitary Association have worked diligently in past years with federal people, to develop uniform methods and rules that meet local conditions and at the same time are sound from the disease-control standpoint. Such a set of Uniform Methods and Rules now has been adopted and published by the United States Agricultural Research Service, Animal Disease Eradication Division, as Bulletin ARS 91-10. Acceptance of these rules by the states has been general, relating to test interpretations, herd and area certification requirements, and animal identification. Rules for the movement of cattle within certified areas and from one certified area in a state to another certified area within the same state have been approved.

In spite of these many areas of agreement on procedures, the requirements for the movement of breeding cattle between states have not been completely settled. While federal regulations have been established, some states have import requirements even more exacting than the federal rules that must be met by sellers of cattle making interstate shipments. This problem of cattle movement is recognized and the hope exists that uniform interstate health regulations approved by all states will be formulated and adopted soon. Many state officials, at present, favor easing some of the restrictions imposed by individual states that are still retarding the free movement of animals across state lines.

### RANGE CATTLE TESTED AT SLAUGHTER

Another recent brucellosis program development that is a step forward, in my opinion, is the blood testing of dry and cull cows on the way to slaughter, rather than on the open range, which is often inconvenient and difficult. Under the plan, a range county can be recertified for another 3-yr. period if at least 15% of the breeding cows going to slaughter centers are blood-tested during the 3-yr. period. Slaughter animals will be tagged to identify their place of origin and, when blood-tested at the market, the herds from which all reactors come will be quickly located and tested. Infected herds discovered by this reactor-tracing procedure will be handled in the customary manner, until infection has been eliminated.

It is said that this new beef cattle market testing program is somewhat comparable in convenience to the milk ring test plan for locating infected dairy herds. However, success will depend a good deal upon the cooperation

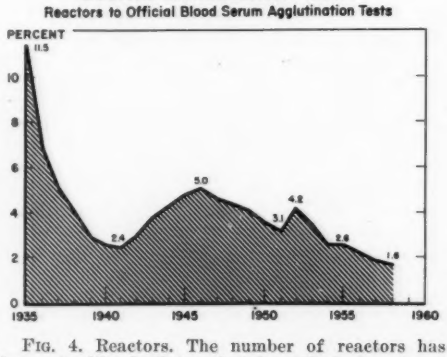


FIG. 4. Reactors. The number of reactors has been steadily declining, but brucellosis is not yet eradicated.

of producers in providing the means for tracing the reactors to the infected herds of origin. Before adoption this plan was first tried with favorable results in range areas in the State of Washington.

### BRUCELLOSIS IN GOATS, SWINE, HUMANS

Often, when thinking of brucellosis eradication, one naturally has cattle in mind as the animal species involved. While the elimination of this disease in cattle is of greatest concern, brucellosis affects other species, including man. It is reported that in 1958, 12,185 goats in 2,320 goat herds received blood agglutination tests. Thirty reactors and 157 suspects were found.

In swine, 2,320 reactors and 3,323 suspects were located among 137,805 hogs in 16,840 herds that were tested. The infection rate was 1.6% reactors and 2.4% suspects. At present, 26 states offer swine brucellosis elimination programs, and the United States Livestock Sanitary Association at its annual meeting last fall suggested a uniform eradication program leading to swine-herd certification for adoption by the states.

To date, swine producers have not become very excited about brucellosis, although some breeders feel it is to their sales advantage to offer stock, especially boars, from certified herds. It appears that as brucellosis is eradicated in cattle, the elimination of the disease in swine will receive increased attention and emphasis. Already, a few states are offering free blood tests to interested swine breeders wishing to test their herds.

As for human brucellosis, the number of cases reported has declined steadily from over 6,000 in 1947 to a little less than 2,000 in 1958. It is believed that the reduced instances of the disease in farm animals during this period have contributed greatly in lessening the infection among humans.

### GUARD AGAINST APATHY AS VICTORY NEARS

In conclusion, it can be emphatically said that brucellosis in cattle is being steadily eliminated upon the farms and ranches of the United States. In the year 1958 alone, 493 additional counties were certified, infected herds declined 21%, and the number of disclosed reactors dropped by 10.8% from the previous year. Today, most large milk markets are receiving milk from brucellosis-free herds only, and thousands upon thousands of cattle upon range have received vaccination protection against this costly disease.

As one looks back 25 yr., to 1934, when brucellosis eradication as a national program took root, it can be seen that progress indeed has been remarkable. The chief danger right now, while we are on the threshold of victory, is that a state of apathy and complacency will develop to retard the final eradication effort. This should be vigorously guarded against, as now is no time to relax, feeling that the job has been completed. Continued cooperation from all quarters is demanded, to finish the task of certifying every county and every state. Even then, eternal vigilance will be required for a long time afterwards, in order that reinfection does not rise to mar the gratifying picture of brucellosis-free herds upon every farm. In the future, as in the past, the American Dairy Science Association can lend its helpful support to this worthy public project.

And lest we not forget, healthy cattle still remain the backbone of a profitable dairy industry.

# OUR ASSOCIATION

## REPORT TO THE AMERICAN DAIRY SCIENCE ASSOCIATION BY ITS REPRESENTATIVE TO THE NATIONAL RESEARCH COUNCIL 1958-1959

W. E. KRAUSS
Associate Director, Ohio Agricultural Experiment Station, Wooster

*Annual Meeting—National Research Council.* High light of the year's activities was the annual meeting of the National Research Council (NRC) which followed the pattern of the highly successful annual meeting of the previous year. Under this plan all eight divisions of the National Research Council met in three plenary sessions. At the first of these sessions presentations were made on the following topics of broad general interest:

Nature and Conduct of Fellowship Programs in Science, discussed by Warren Weaver.

U. S. Science in Foreign Aid, discussed by George Harrar.

Means and Patterns for the Support of Scientific Research, discussed by Alan Waterman.

The Place of Behavioral Science in the National Research Council, discussed by Clyde Kluckholn and Don Marquis.

*Annual Meeting—Division of Biology and Agriculture, Burr Steinbach, Chairman.* All 34 of the member scientific societies had representatives present. For the benefit of new representatives, and for the orientation of others, Steinbach outlined the function of the members and the relationship of their societies to the over-all National Academy–National Research Council structure.

The principal function of members of the Division is to examine its work and to try to guide it along the lines of greatest need, interest, or importance to various branches of biology and agriculture, and to science, government, the national welfare, and human welfare in general. Many of the members of the Division also participate in the work of its boards, committees, and panels, through which the experience and wisdom of many American biologists are brought to bear on problems in biology and agriculture.

The Academy–Research Council is enjoined, when called upon, to advise the government, but is completely outside of government control. This is the feature distinguishing it from other advisory agencies, created since World War II (National Science Foundation, President's Science Advisory Committee, etc.). Thus, the NAS-NRC has evolved from a passive body to an aggressive one; it must determine the role it should play in the national and international scene.

The Executive Committee has asserted that the Division must take the lead or participate in broad studies dealing not only with biology or agriculture as natural sciences, or not only with natural sciences extending beyond the boundaries of biology and agriculture into the subjects of other divisions, but with natural and social sciences connected with agriculture and human welfare. The general problem of renewable natural resources is an example, as is that of educational policies conducive to the production of scholars in biology.

W. E. Krauss, Chairman of the Agricultural Board, in keeping with the newer outlook previously expressed, anticipated broadening the scope of the Board's activities to encompass a variety of problems having social and economic aspects, such as:

Population dynamics, land dynamics, and the manpower needed for future production.

Effect of limited or total war on agriculture—decontamination and then restoration of production.

Effect of increasing mechanization and food consumption habits on patterns of production.

Effect of U. S. agriculture on foreign agriculture, and vice versa.

Impact of all factors on the development of a sound agricultural research program.

A grant of $50,000 has been made by the Rockefeller Foundation to support the above program. Applications are encouraged from scientists who may wish to devote two to three years to spearheading and coordinating the work to be done.

*Agricultural Research Institute.* President Burt Johnson emphasized the unique intermingling among land-grant institution personnel, USDA, and other government agencies, and officials from industry made possible through membership in ARI. Development of forums, advising and helping finance the Agricultural Board, constitute the principal activities of ARI.

*Food and Nutrition Board.* LeRoy Voris, Executive Secretary, related the establishment of dietary standards, flour enrichment, encouragement of international studies on protein malnutrition, and creation of the Food Protection Committee, as outstanding accomplishments of this Board.

*Food Protection Committee.* Paul Johnson, Executive Secretary, announced that steps were under way to hold a conference on toxicology, in cooperation with the Committee on Toxicology of the Division of Chemistry and Chemical Technology, for the purpose of promoting toxicology as a scientific discipline separate from pharmacology.

*Institute of Laboratory Animal Resources (ILAR).* Chairman D. W. Jenkins pointed to the establishment of minimum standards for mice; sponsorship of the Animal Breeders Association for maintenance of standards; establishment of a new committee on cell and tissue culture; and participation in international surveys of laboratory animals, as outstanding accomplishments. The ILAR Washington Office has become an effective information center in this field.

In the general discussion following presentation of the previous reports, the question of usefulness of the Division was raised. Under the new aggressive general policy it was felt that the NRC could continue to have marked influence in guiding major policies affecting scientists. Greater participation, through suggestions by scientific society representatives, and greater recognition by the societies themselves of the potentials of the NRC, would help make this possible.

To this end, a monthly newsletter to all society representatives will be forthcoming. A questionnaire has been sent to each Society, the answers to which will be helpful in bringing the societies and the divisions closer together than does attendance alone by the representative at the annual meeting.

A matter of considerable concern to the Division is the broad problem of use and care of renewable natural resources. It is felt that a standing committee is needed to stimulate basic research in this field and consider from the scientific point of view public policy relating to renewable natural resources. Member society opinion regarding the role of NAS-NRC in this field is solicited.

### ACTIVITIES DURING THE YEAR

Members of the Division participated in the procurement of nominees for a U. S. National Committee on the International Union of Biological Sciences. Included in the personnel of this new committee is J. L. Lush, a member of our Society.

Productivity of the Division, as in all scientific endeavors, can be measured in part by publications. These were numerous during the year (see the appendix). Arrangements for procural of the publications listed can be made through Frank L. Campbell, Executive Secretary, Division of Biology and Agriculture, National Academy of Sciences, 2101 Constitution Avenue, Washington 25, D. C.

## APPENDIX

### Status of Publications Since Annual Meeting, 1958

| PUBLISHED | IN PREPARATION |
|---|---|

#### HANDBOOKS ON BIOLOGICAL DATA

Handbook on Toxicology
- Vol. III, Insecticides
- Vol. IV, Tranquilizers
- Vol. V, Fungicides

Handbook on Respiration

Handbook on Circulation

#### BIOLOGY COUNCIL

The Developmental Biology Conference Series, 1956:
- Embryonic Nutrition
- Regeneration in Vertebrates
- Cytodifferentiation
- Environmental Influences on Prenatal Development
- Immunology and Development
- Physiology of Insect Development
- Dynamics of Proliferating Tissues
- Endocrines in Development
- Mitogenesis
- Wound Healing and Tissue Repair

Concepts of Biology (NAS-NRC reprint)

(APPENDIX — Cont'd)

| Published | In Preparation |
|---|---|

### Educational Policies

Biological Education—A Partial Bibliography

Recommendations on Undergraduate Curricula in the Biological Sciences

Improving College Biology Teaching

Criteria for Preparation and Selection of Science Textbooks

Career Opportunities in Biology (NAS-NRC reprint)

Laboratory and Field Studies in Biology: A Sourcebook for Secondary Schools

### Food and Nutrition Board

The Role of Dietary Fat in Human Health

Dietary Fat and Human Health: A Summary Based on the Role of Dietary Fat in Human Health

Recommended Dietary Allowances

Cereal Enrichment in Perspective

Proceedings, Vol. XVIII (Mimeographed)

Food Packaging Materials—Their Composition and Uses

The Safety of Polyoxyethylene (8) Stearate for Use in Food

Insignificant Levels of Chemical Additives in Food Food and Drug Administration, *Cosmetic Law Journal*, **15**: 477-479

Evaluation of Protein Nutrition with Emphasis upon Amino Acid Proportionalities

Revision of:
"The Use of Chemical Additives in Food Processing"

Revision of:
"Principles and Procedures for Evaluating the Safety of Intentional Chemical Additives in Foods"

### Committee on Photobiology

Research in Photosynthesis
The Gatlinburg Conference, October 25-29, 1955

In press:
Photoperiodism and Related Phenomena in Plants and Animals. The Gatlinburg Conference, October 29–November 2, 1957

### Institute of Laboratory Animal Resources

Quarterly News Letter: Information on Laboratory Animals for Research

Animals for Research: A Catalogue of Commercial Sources

A Handbook of Laboratory Animals

Standards for the Transportation of Laboratory Dogs

### Agricultural Board and ARI

Research Needs Relating to Agricultural Equipment (Reprint)

Whither Weather, Climate and Agriculture (Reprint)

Nutrient Requirements for Beef Cattle (Revised)

Composition of Cereal Grains and Forages

Races of Maize in Brazil and Other Eastern South American Countries

Proceedings, Seventh Annual Meeting, ARI

U.S.–Canadian Tables of Feed Composition

Methods for the Examination of Poultry Biologics

Methods in Range Research

# BOOK REVIEW

INVESTIGATION OF MILK PROTEINS. P. F. D'Achenko. Proceedings of the "Federative Scientific Research Institute of the Dairy Industry (VNIMI)", No. 19, Moscow, 1959. 85 pp., 25 figs., and 38 tables. In Russian. Broche price, 4 Rb. 90 Kop.

This booklet summarizes the results of milk protein research carried out by the members of the above-mentioned institute, together with a review of non-Russian literature on the subject.

The book's title, however, suggests a broader scope than is given by its real content, for 79 pages of the book are devoted to the chemistry of casein; whereas, the remaining six must complete the picture of the whey proteins, of enzymes and low-molecular protein material in milk, and of the protein composition of the fat globule membrane. It is clear that this is by no means a simple task, and that the author failed to accomplish it. As a general survey of our knowledge of the milk proteins, the present review can not sustain a comparison with other treatments of the same subject; for instance, with McMeekin's recent review (Neurath-Bailey, *The Proteins*, Vol. IIA). Rather, it should be considered as a report of the author's own contributions to the chemistry of casein, completed with some results from literature.

Nevertheless, D'Achenko's treatment of casein chemistry is worth while, to be studied by non-Russian, as well as Russian, workers interested in this field.

The book is divided into three chapters: I. Classification of milk proteins; II. Dispersive properties of milk proteins; III. Coagulation of milk proteins.

Chapter I deals with such subjects as heterogeneity, amino acid composition, and titration of casein. The reactions of casein with aldehydes and especially with aldoses are treated extensively.

In Chaper II the state of dispersion of the casein micelles in milk has been studied by the author mainly by light-scattering and electron microscopy. The established distribution of particle dimensions is found to be in fair accordance with the results of other workers.

In Chapter III an extensive comparison is made of the coagulations of the milk proteins by acid, by rennet, and by calcium ions. It is suggested that the precipitation of the milk proteins by calcium ions at 95° C. may find industrial application, because of its completeness.

Finally, the following comments should be made:

Paper electrophoresis of casein can be achieved also without the addition of large quantities of urea. In this connection, the casein composition resulting from the Russian experiments (32.5% $\alpha$, 67.5% $\beta$), therefore, seems highly improbable and certainly does not agree with the results obtained by other workers and by different techniques.

Whether in this instance, as well as in others, the ionic strength of the casein solutions during titration is kept constant remains obscure.

The molecular weights of casein and paracasein, as revealed by light-scattering in 40% urea solution, bear practically no relation to the state of casein, respective of the paracasein in the milk. It is uncertain, therefore, that such measurements could afford some insight into the changes of casein brought about by rennet.

The arguments for a phospho-amidaze action of rennet brought forward by D'Achenko are not very convincing. In fact, they contradict rather than substantiate such an action.

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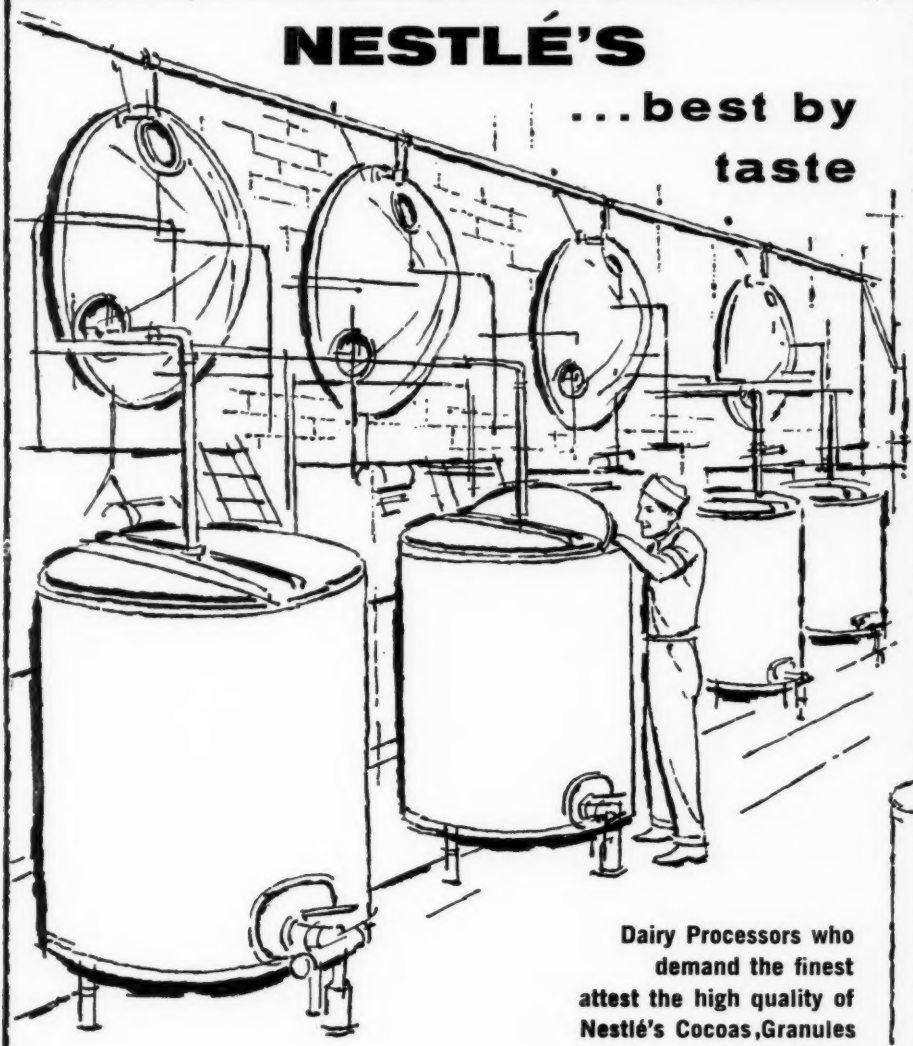

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